Functional investigation of receptor-like kinase domains and motifs crucial for root nodule symbiosis

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Erklärung

Hiermit erkläre ich, dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist. Ich habe nicht versucht, anderweitig eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen.

München, den 04.04.2025

Anna Isabel Seidler

Table of Contents

Abbreviation index

AM	Arbuscular mycorrhiza
ARID	AT-rich interaction domain
ATP	Adenosine triphosphate
AVG	aminoethoxyvinylglycine
BAK1	Brassinosteroid insensitive 1-associated receptor kinase 1
BRI1	Brassinosteroid insensitive1
CCaMK	Calcium- and calmodulin-dependent serine/threonin kinase
CEP	C-terminally encoded peptide
CERK1	Chitin elicitor receptor-like kinase1
CFTR	Cystic fibrosis transmembrane conductance regulator
CLE	Clavata3/embryo surrounding region-related
CLV	Clavata
CO	Chitooligosaccharide
Co-IP	Co-immunopurification
CRA2	Compact root architecture 2
C-term	C-terminus
DELLA	Aspartic acid–glutamic acid–leucine–leucine–alanine
DFG-motif	Aspartate-Phenylalanine-Glycine-motif
DMI1	Does not make infections1
DMI2	Does not make infections2
DMI3	Does not make infections3
Ds	*Discosoma spec*.
ED	Extracellular domain
EDDHA	Ethylenediamine-N,N′-bis(2-hydroxyphenylacetic acid)
ERN1	Ethylene responsive factor required for nodulation 1
Ev	Empty vector
FaFaCuRo	Fabales, Fagales, Cucurbitales, Rosales
Flg22	Flagellin 22
FLIM-FRET	Fluorescence lifetime imaging microscopy - Förster resonance energy transfer
FLOT4	Flotillin 4
FLS2	Flagellin-sensing2
FP-Medium	Farhaeus plant medium
HAR1	Hypernodulation Aberrant Root formation 1

HMGR	3-Hydroxy-3-methylglutaryl coa reductase
ID	Intracellular domain
IPD3	Interacting protein of dmi3
JXT	Juxtamembrane
KD	Kinase domain
LB	Lysogenic broth
LCO	Lipochitooligosaccharide
Lj	*Lotus japonicus*
LRR	Leucin-rich repeat
LYK1	LysM domain-containing receptor-like kinase 1
LYK3	LysM domain-containing receptor-like kinase 3
LYK10	LysM domain-containing receptor-like kinase 10
LYK13	LysM domain-containing receptor-like kinase 13
LysM	Lysine motif
MAMP	Microbe associated molecular pattern
MAPKK	Mitogen-activated protein kinase kinase
MES	2-(N-morpholino)ethanesulfonic acid
miR	microRNA
MLD	Malectin-like domain
Mt	*Medicago truncatula*
NFP	Nod-factor perception
NFR1	Nod-factor receptor 1
NFR5	Nod-factor receptor 5
NF-YA	Nuclear Factor YA
NiCK4	NFR5-interacting cytoplasmic kinase
NIN	Nodule inception
Nod-factor	Nodulation factor
Nod-genes	Nodulation genes
NORK	Nodulation receptor kinase
NSP1	Nodulation signaling pathway1
NSP2	Nodulation signaling pathway2
NUP133	Nucleoporin133
NUP85	Nucleoporin85
Os	*Oryza sativa*
Pan	*Parasponia andersonii*
PCR	Polymerase chain reaction
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)

proUbi	Ubiquitin promoter of *L. japonicus*
PUB1	Plant-U-box protein1
PUB2	Plant-U-box protein2
RAM1	Reduced arbuscular mycorrhiza1
RAM2	Reduced arbuscular mycorrhiza2
RAxML	Randomized Axelerated Maximum Likelihood
RING	Really interesting new gene
RINRK1	Rhizobial infection receptor-like kinase 1
RLK	Receptor-like kinases
RNAi	RNA interference
RNS	Root nodule symbiosis
SIE3	SymRK interacting E3 ligase
SINA	Seven in absentia
SIP	SymRK interacting protein
Sl	Solanum lycopersicum
SUMO	Small ubiquitin-like modifier
SYMREM	Symbiosis specific remorin
SymRK	Symbiosis receptor-like kinase
TM	Transmembrane domain
TMID	Transmembarne domain and intracellular domain
TML	Too much love
TOPO	Topoisomerase
YAQ-motif	Tyrosine, alanine, glutamine-motif

Contributions to this study

The experiments underlying Fig.1, Fig. 2, Fig. 5, Fig. 6, Fig. 7, Fig. 8, and all supplementary figures were fully designed, planned and executed by me.

The constructs for Fig. 3 (also appearing in other figures) were designed and produced by Martina Ried (MR) with assistance in cloning by me. The experiment underlying Fig. 3 was performed by MR, the data were plotted and analyzed by me. I also performed a verification of one construct.

The overview slides of Fig. 4 were provided by MR, the histological sections were performed by me.

The *in silico* design of the constructs for Fig. 9 and Fig. 11 was performed by MR. Cloning and the experiment itself as well as data analysis was performed by me.

The construct *proUbi:SymRK* from Fig. 10 was designed and produced by MR. The experiment and the constructs *proUbi:SlLYK10* and *proUbi:NFR5* were performed by me.

Abstract

Arbuscular mycorrhiza (AM) symbiosis is a widespread, ancient symbiosis that facilitates nutrient uptake by land plants. In contrast, the intracellular uptake of nitrogen-fixing bacteria is rare and only occurs in a distinct phylogenetic clade, comprising the Fabales, Fagales, Rosales and Cucurbitales (FaFaCuRo clade). While a big variety of Fabales engage in root nodule symbiosis (RNS) with nitrogen-fixing bacteria, it is rare among the other members of the FaFaCuRo clade. This led to the assumption, that there was predisposition event at the root of this clade. The establishment of these two symbioses requires a set of shared genes, the so-called common symbiosis genes. Among them, is the Symbiosis Receptor-like Kinase (SymRK). SymRK homologues from the Eurosids I clade were described to complement RNS and AM symbiosis in the model species *Lotus japonicus symrk* mutant background, whereas SymRK from species outside that clade only complemented the AM symbiosis phenotype but not the RNS.

This study focusses on the differences in complementation capacity of SymRK from *Solanum lycopersicum* (Tomato SymRK) and *L. japonicus* (Lotus SymRK), a Fabales species, to get a better understanding of SymRK evolution. We found that, in contrast to published data, the complementation of the *L. japonicus symrk* mutant with Tomato SymRK exhibited rare formation of infected nodules and very abundant primordia-like, but uninfected structures, that we named swellings. We wanted to clarify which domain of Lotus SymRK allows the protein to convey its full complementation capacity, i. e. abundant nodule without swelling formation. Therefore, we performed domain swap experiments by swapping the subdomains of the extracellular domain, the transmembrane domain and the intracellular domain. This experiment led to the conclusion that the intracellular domain plays an important role for the complementation capacity. Even though ubiquitination is known to play an important role for SymRK function, I did not observe differences in complementation capacity of Tomato SymRK and Lotus SymRK predicted ubiquitination site switch mutations. A further approach to come closer to the responsible amino acids was another domain swap experiment, swapping the intracellular domain in four different parts according to their amino acid conservation. This experiment though revealed that the role of the transmembrane domain or the extracellular domain is more important than suggested by the first domain swap experiment. Furthermore, the postulated evolutionary conservation of the intracellular domain of Nod-factor-receptors of *Lotus japonicus (Lj) Lj*NFR1 and *Lj*NFR5 with their homologues from *Solanum lycopersicum (Sl) Sl*LYK1 and *Sl*LYK10 could be observed. Taken together these results suggest distinct and complex evolutionary paths of *Lotus japonicus* receptor-like kinases enabling root symbiosis.

Introduction

Macronutrients in plant nutrition and agriculture

Land plants are dependent on the uptake of nutrients from the soil with the help of their roots. These nutrients provide osmotic balance, necessary components of plant proteins, nucleic acids or function as co-factors of enzymes (Raven et al. 2013). Plant nutrients can be categorized in macronutrients and micronutrients. Macronutrients are necessary in relatively big amounts up to 1000 mg per kg dry weight, whereas micronutrients are only needed in small amounts of less than 100 mg per kg dry weight or even only trace amounts (Raven et al. 2013). The nutrients with the highest required amount include nitrogen and phosphorus, thus often used in fertilizer. For plants to take it up, nitrogen must be present in the form of ammonia or nitrate (Xu et al. 2012). Therefore, the very abundant molecular nitrogen of the air is not available to the plants due to its very low chemical reactivity (Robertson and Vitousek 2009). In agricultural settings, the plants are consequently fertilized with ammonia or nitrate either from compost, manure or with synthetic nitrogen fertilizer (Xu et al. 2012). All these forms of nitrogen supply to the roots bear the risk of overfertilization with the risk of leaching in surrounding waters leading to eutrophication and the emission of N_2O with its impact on climate change and human health (Robertson and Vitousek 2009; Thompson et al. 2019). In addition, the technical nitrogen fixation by the Haber-Bosch process to produce ammonia from molecular nitrogen is very energy demanding. It is estimated that around 2% of the world-wide natural gas per year is used as hydrogen and energy source for the Haber-Bosch process emitting 1.2 % of the entire anthropogenic CO_2 (Cherkasov et al. 2015; Smith et al. 2020).

Another very important macronutrient is phosphorus, taken up by plants in the form of phosphate, also frequently used in fertilizers. Mineral phosphate can be obtained from mining, however easily accessible sites with high amounts of mineral phosphate are limited. Estimations predict that the mineable phosphate rock will run out within the next decades to centuries (Gilbert 2009; Vaccari et al. 2019). In addition, phosphate reserves are highly concentrated with over 70 % in a single country: Morocco, with the diplomatically not acknowledged region of Western Sahara. This can lead to important future economic challenges and geopolitical questions on world-wide phosphate availability (Cooper et al. 2011). Thus, to ensure food security and environmental sustainability, there is a great need to reduce fertilization.

Arbuscular mycorrhiza – ancient and widespread

The adaption to life on land by plants required an adaption of water and nutrient uptake from the surrounding water to uptake from the soil via roots. In the process, roots evolved to form a fine network in the soil (Raven et al. 2013). To further improve nutrient and water uptake, plants engage in close relationships with microorganisms, mainly fungi and bacteria (Raven et al. 2013). Root associated fungi expand the surface for nutrient and water uptake. Among these interactions, the symbiosis with so called arbuscular mycorrhiza fungi (AM) is very ancient. In fossils of very early plants, the typical tree shaped structure of the fungus inside root cells can be observed (Remy et al. 1994). The AM provide the plants with water and nutrients like phosphate from areas plant roots cannot reach and the plants provide the fungi in return with carbohydrates and lipids (Shachar-Hill et al. 1995; Bago et al. 2000; Jiang et al. 2017; Keymer et al. 2017; Luginbuehl et al. 2017). The ability to interact with arbuscular mycorrhiza fungi is wide spread among the plant kingdom from liverworts of the genus *Marchantia* to the majority of flowering plants (Russell and Bulman 2005; Parniske 2008). Only few plant genera secondarily lost this ability, among them the frequently used model plant *Arabidpsis thaliana* (Delaux et al. 2014).

All AM fungi belong to one phylogenetic group, the Glomeromycota, and most species of that phylum are obligate biotrophs engaging in AM symbiosis (Schüßler et al. 2001). AM requires the fungus to surpass the epidermis of the host plant's root and infect the endodermal cells intracellularly, where it forms tree like structures, called arbuscles (Parniske 2008). To allow that intraradical and intracellular infection, a tightly regulated program is necessary that protects the plants from infection with pathogenic microorganism and, at the same time, accommodates the symbiotic fungus (Pimprikar and Gutjahr 2018).

Intracellular accommodation of bacteria in root nodules in the FaFaCuRo clade

Bacteria can associate with plants as well. On one hand, they can live in the plants' rhizosphere and produce metabolites or fix inorganic nitrogen to ammonia in close vicinity to the roots, so plants can take advantage of those chemicals (Nag et al. 2020). This type of bacterial plant interaction is widespread and is known to contribute to plant health and nutrition (Nag et al. 2020). On the other hand, bacteria can also engage in endosymbiosis with plants. In contrast to the monophyletic AM fungi, the bacteria involved in uptake into plant cells are very diverse and include cyanobacteria, alphaproteobacteria and actinomycetes (Parniske 2018). Of those bacterial-plant endosymbioses, the so-called root nodule symbiosis (RNS) plays an ecologically and economically very important role. RNS featuring intracellular uptake of nitrogen-fixing bacteria in living root cells is only known in a few plant families, the Fabales, Fagales, Rosales and Cucurbitales all belonging to one monophyletic clade (FaFaCuRo)

(Soltis et al. 1995; Griesmann et al. 2018; Parniske 2018). The bacteria are accommodated in newly formed organs. These so called nodules usually derive from dividing inner cortical, but also rarely pericycle cells upon bacterial infection (Libbenga and Harkes 1973; Yang et al. 1994; Xiao et al. 2014). Intracellular uptake of bacteria into nodules varies between the different plant families capable of this type of symbiosis: While plants of the Fabales interact with a gram negative group of bacteria called Rhizobia, in Fagales, Rosales and Cucurbitales the interaction takes usually place with the gram positive Frankia group of bacteria. There is only one exception: *Parasponia andersonii*, which interacts with Rhizobia, even though belonging to the Rosales (Lindström and Mousavi 2010; Griesmann et al. 2018). Root nodule symbiosis is widespread among Fabales, but remains the exception for genera or species of the Fagales, Rosales and Cucurbitales (Franche et al. 2009). Thus, there are phylogenetic indications, that there is only one origin of this type of symbiosis and that there might have been a predisposition event in the Eurosid I clade, comprising the FaFaCuRo clade and some more orders including the Brassicales. to prime those plant families for the new symbiotic interaction (Soltis et al. 1995; Griesmann et al. 2018; van Velzen et al. 2018; Cathebras et al. 2022). Interestingly, the accommodation of bacteria in RNS resembles the accommodation of AM fungi including their chemical communication with the plant (Harris et al. 2020).

Chemical communication between symbionts and plants

Plants are constantly subjected to microbes, among them pathogens, commensals, beneficial microbes and intracellular mutualistic microbes. Thus, plants evolved a sophisticated set of receptors, which detect microbe associated molecular patterns (MAMPs). Those can be parts of microbial cell walls, flagella, necessary components of their metabolism or secreted molecules (Shu et al. 2023). Interestingly many commensal microbes have evolved MAMPs such as the flagellin epitope flg22, that evade detection and immune system (Colaianni et al. 2021). In addition, plant defense responses are confined to regions of microbial perception coupled with cellular damage (Zhou et al. 2020; Tsai et al. 2023). A colonization of the root by mutualistic microbes however requires a dedicated recognition of the symbiont by a defined chemical cross-talk. Thus, plants enrich the rhizosphere with beneficial microbes by excreting chemicals, so called root exudates inducing growth, motility and metabolic responses in AM fungi or root endosymbiotic bacteria (Bais et al. 2006).

To establish AM symbiosis plants secret strigolactones into the rhizosphere, which trigger germination, hyphal growth towards the roots and secretion of lipochito-oligasaccharides (LCOs) and chito-oligosaccharides (COs) of the fungi (Akiyama et al. 2005; Besserer et al. 2006; Maillet et al. 2011; Genre et al. 2013). These substances are also called Myc-LCOs and Myc-COs respectively. For RNS, the plant exudates flavonoids, which trigger the induction of

the so-called nod-genes in Rhizobia, that, among others, are required for Nod-factor synthesis (Peters et al. 1986; Abdel-Lateif et al. 2012). The Nod-factors of different Rhizobia are LCOs with specific decorations, leading to an interaction specificity of certain legumes with sometimes only one species of bacteria (Ghantasala and Roy Choudhury 2022). Also in Frankia bacteria, canonical *nodABC* genes for the production of LCOs were detected in the genome, suggesting a potential role during symbiosis (Persson et al. 2015; Nguyen et al. 2016). Both the COs and LCOs are recognized by the plant by receptor-like kinases containing LysM motifs. These motifs consist of 44-65 amino acids and are known to bind chitin and peptidoglycans (Buist et al. 2008)

For Rhizobia, the receptors responsible for Nod-factor perception are called Nod-Factor Receptor 1 in the model legume *Lotus japonicus* (*Lj*NFR1) and *Mt*LYK3 in the second model legume *Medicago truncatula* (Radutoiu et al. 2003; Smit et al. 2007). This receptor consists of three LysM-domains, a transmembrane domain and a functional kinase domain. A second receptor involved in the perception of Nod-factor is Nod-Factor Receptor 5 in *L. japonicus* (*Lj*NFR5) and Nod-factor perception in *M. truncatula* (*Mt*NFP). They are both necessary for nodule establishment and their overexpression triggers formation of nodules in the absence of symbionts (Ried et al. 2014). Thus, mutants of these receptor do not show any bacterial entry or nodule formation upon inoculation with compatible bacteria in many legume species (Radutoiu et al. 2003). The intracellular accommodation of AM fungi was not impaired in an initial study (Wegel et al. 1998), however in a more recent study, a reduction of AM colonization was detected for *nfr1*, but not *nfr5* mutants (Zhang et al. 2015). Both receptors directly bind Nod-factor on the extracellular domain with high affinity (Broghammer et al. 2012).

A double mutant of the two closest homologues to *Lj*NFR5/*Mt*NFP in *Parasponia andersonii* has been shown to be impaired in RNS as well, but not in the establishment of AM symbiosis. In contrast the two closest homologues of *Lj*NFR1/*Mt*LYK3 were impaired in both RNS and AM (Rutten et al. 2020). Interestingly, a RNAi silencing approach which targeted the *Pan*NFP intracellular domain also affected the interaction with AM fungi and impaired arbuscle formation (Op Den Camp et al. 2011).

In other species, other LysM receptors with high similarity to *Lj*NFR1 and *Lj*NFR5 have been identified to play a role in the establishment of AM symbiosis. In rice (*Oryza sativa, Os*), mutants of the receptor CHITIN ELICITOR RECEPTOR KINASE (*Os*CERK1) are impaired in the interaction with AM fungi (Zhang et al. 2015). Interestingly, *Os*CERK1 and its homologues in other species, including *Arabidopsis thaliana* which does not engage in AM symbiosis, play an important role in plant immunity by detecting fungal cell wall components also consisting of chito-ologosaccharides (COs) (Miya et al. 2007; Shimizu et al. 2010). In many species, it seems that CERK1 homologues have a dual role in symbiosis and defense against pathogens

(Miyata et al. 2014). In tomato, there is not only one homologue of *Lj*NFR1 and *Os*CERK1, but there are four paralogues described with different functions in AM and immunity (Liao et al. 2018). Therefore, the formerly called *Sl*CERK1 was renamed in *Sl*LYK1 (Miyata et al. 2014; Liao et al. 2018). *Sl*LYK10, a homologue of *Lj*NFR5 in tomato (*Solanum lycopersicum*) plays an important role in the establishment of AM symbiosis and can directly bind Myc-factor (Buendia et al. 2016; Girardin et al. 2019). In legumes, an expansion of LysM-receptor genes has been described (Lohmann et al. 2010). Therefore, likely due to redundancy, no receptor mutant with a strong impairment in AM establishment could be identified to date. The receptor *Mt*LYK9, a homologue of *Os*CERK1 from *M. truncatula* has been identified to play a crucial role in plant immunity restricting pathogen growth as well as promoting AM symbiosis (Gibelin-Viala et al. 2019).

The specificity of the LysM-receptors lies in their extracellular domain, where they bind molecules with a high specificity. Already small differences in the side chain decoration of a Nod-factor can result in different signaling outputs (Bisseling and Geurts 2020; Bozsoki et al. 2020). This leads to the very high specificity in legumes to a specific rhizobial symbiont. The intracellular domains of these receptors are often functionally conserved between different species. For instance, *L. japonicus* Nod-LCO receptor mutant plants expressing a hybrid construct of the *Lj*NFR1 and *Lj*NFR5 with the extracellular domains of the *L. filicaulis* NFR1 and NFR5 receptors and the intracellular domains of the *L. japonicus* receptors extended the host range of *L. japonicus* (Radutoiu et al. 2007). This even applies for more distantly related plant species. For example, hybrid receptors of extracellular domains of *Lj*NFR1 and *Lj*NFR5 with the intracellular domains of their homologues in rice can complement the respective mutant phenotypes of these receptors in the legumes with the intracellular uptake of bacteria and the development of a nodule (Miyata et al. 2016).

AM and RNS share a set of genes necessary for intracellular infection of AM and nitrogen-fixing endosymbionts as well as nodule organogenesis

The perception of Myc-LCOs, Myc-COs and Nod-LCOs leads to very similar early responses in both nodulation and AM establishment. A hallmark of both symbioses is the triggering of a oscillating calcium influx in the nucleus, called calcium spiking, after activation of the above described LysM-receptors (Sieberer et al. 2009; Genre et al. 2013). To achieve the calcium spiking and potential to decode it, several genes are necessary, and mutants of these genes are not able to engage with either AM fungi or nodulating bacteria. Therefore, these genes are called common symbiosis genes (Kistner and Parniske 2002; Oldroyd 2013).

Among the common symbiosis genes, there is a receptor-like kinase, called Symbiosis Receptor-like Kinase (*Lj*SymRK) in *L. japonicus*, Does Not Make Infections 2 in *M. truncatula* (*Mt*DMI2) and Nodulation Receptor Kinase (*Ms*NORK) in alfalfa (*Medicago sativa, Ms*) (Catoira et al. 2000; Endre et al. 2002; Stracke et al. 2002). Moreover, the activation of calcium oscillation is dependent on 3-Hydroxy-3-Methylglutaryl CoA Reductase 1 (HMGR1) and its production of mevalonate (Kevei et al. 2007). For the signal transduction from the receptor level to the nucleus, the involvement of cytoplasmic kinases has been proposed as well (Chen et al. 2012). In *Lotus japonicus,* the NFR5-interacting cytoplasmic kinase (NiCK4) has been identified to interact with *Lj*NFR5 and to play a role in the nodulation process (Wong et al. 2019).

Furthermore, several cation channels are essential for calcium-spiking. In *L. japonicus,* the cation channels *Lj*CASTOR and *Lj*POLLUX have been identified to be essential for the generation of calcium oscillations, whereas in *M. truncatula Mt*DMI1 can take over the role of both *Lj*CASTOR and *Lj*POLLUX (Peiter et al. 2007; Charpentier et al. 2009). These channels are located in the nuclear envelope, where they access the endoplasmatic calcium storage in the perinuclear space. It is under debate whether these receptors are direct calcium channels, or if they are potassium ion channels and their role is to provide counterions for the calcium flux mediated by different channels e.g., from the cyclic nucleotide gated channel family (CNGC15s) (Charpentier et al. 2009; Kim et al. 2019). In particular, CNGC15 forms complexes with *Mt*DMI1 and is permeable for Ca^{2+} ions (Charpentier et al. 2016; Liu et al. 2022). The stabilization of calcium oscillation is also dependent on the ATPase MCA8 localized in the nuclear envelope (Capoen et al., 2011). Furthermore, parts of the nuclear pore (NENA, NUP85, and NUP133) are also essential for the generation of calcium oscillation (Saito et al. 2007; Binder and Parniske 2013).

Another factor necessary for nodulation and AM establishment is Calcium- and Calmodulin-dependent serine/threonin kinase *(Lj*CCaMK) in *L. japonicus* or Does Not Make Infection 3 (*Mt*DMI3) in *M. truncatula* (Lévy et al. 2004; Mitra et al. 2004; Tirichine et al. 2006). This kinase has an autoinhibition domain, which prevents kinase function in the absence of calcium signals (Takeda et al. 2012). After binding of calcium and calmodulin, this kinase becomes active and phosphorylates its targets (Tirichine et al. 2006). The transcription factor *Lj*Cyclops/*Mt*IPD3 is among the proteins phosphorylated by CCaMK (Yano et al. 2008). Cyclops activates the expression of several genes necessary for the infection of plant roots with bacteria or AM fungi. Therefore, it is suggested that the CCaMK-Cyclops complex is “decoding” the calcium spiking (Singh et al. 2014). Finally, Nodulation Signalling Pathway 2 (NSP2) is another transcription factor, necessary for the initiation of both symbioses (Catoira et al. 2000; Murakami et al. 2006).

After the activation of Cyclops, the signaling cascade of nodulation and AM symbiosis diverges. For the initiation of nodulation, the expression of *NIN* and *ERN1* is activated with the help of NSP1 (Cerri et al. 2012, 2017). The AM specific gene *RAM2* is transcriptionally activated after stimulus by Myc-LCOs and Myc-COs with a complex of Cyclops/DELLA/RAM1 binding the *RAM2*-promoter (Pimprikar et al. 2016).

The early physiological responses following the calcium spiking are also very similar in nodulation and AM symbiosis: the nucleus repositions to the apical part of the epidermal cell (i.e. the root hair for nodulation, or an ordinary epidermal cell for AM, respectively) (Sieberer et al. 2009). Afterwards, the cell wall weakens to allow an invagination of the membrane, forming a pre-penetration apparatus or pre-infection thread in AM or nodulation, respectively (van Brussel et al. 1992; Genre et al. 2005). For the formation of these structures and their growth into the accommodation structures, there is the need of factors involved in vesicle transport. They are believed to deliver the materials for the growth of the membrane compartment containing the respective microbe (Harrison and Ivanov 2017).

The AM fungi and bacteria are thus always surrounded by a plant membrane. They grow in these plant-made membrane strings into the cortical part of the root, where the AM fungi ramify in the arbuscle shape and the membrane enclosed bacteria are released into the cytoplasm (Gutjahr and Parniske 2013; Parniske 2018).

Domains of SymRK and their function

Even though SymRK and its crucial role is already known for more than 20 years and intense research efforts by several groups, its precise and undisputed function in the establishment of symbiosis has not been elucidated (Holsters 2008; Sanchez-Lopez et al. 2012). However, some conclusion can be drawn by looking at the different structural domains and interactors. As many transmembrane receptors, SymRK has its N-terminus facing the extracellular space whereas the C-terminus stays intracellularly (Markmann et al. 2008). SymRK has an extracellular domain consisting of a malectin-like domain, and a leucin-rich repeat (LRR) domain (Antolín-Llovera et al. 2014b; Singh and Verma 2023). Malectin domains (MD) are known to bind carbohydrates in animals, but the exact function of malectin-like domains (MLD) in plants is still unclear. It is speculated, that malectin-like domains might interact with the carbohydrates of the plant cell wall (Yang et al. 2021). A defined role for the malectin-like domain of SymRK has not been established yet, especially as it is cleaved off, at least in the setting of SymRK overexpression (Antolín-Llovera et al. 2014b). The purpose of the cleavage is not understood either.

LRR domains bind many different kinds of ligands and are therefore often part of immunity receptors like Toll-like receptors in mammals or flagellin-sensing 2 (FLS2) in plants (Gómez-Gómez and Boller 2000; Bell et al. 2003). These receptors consist of 19-25 (animals) and 16-28 repeats (plants) of LRRs, respectively which form a horseshoe-like structure that binds the respective ligand (Bell et al. 2003; Robatzek and Wirthmueller 2013). In SymRK, there are only two or three repeats of the LRRs (Markmann et al. 2008), a ligand-binding comparable to FLS2 is therefore unlikely. The LRRs might rather be part of the interaction surface for other proteins like NFR5 (Antolín-Llovera et al. 2014b).

In addition, the single transmembrane domain consisting of an α-helix of hydrophobic amino acids, might play a role in interactions with other receptors or other membrane bound proteins (Lefebvre et al. 2010; Antolín-Llovera et al. 2014b). Adjacent to the transmembrane domain, the so called juxtamembrane domains can be found intracellularly and extracellularly. These parts of receptors have not been visualized as a folded part of the protein in X-ray crystallography, but also potentially play a role in protein ubiquitination and endocytosis (Dávila-Delgado et al. 2023).

The intracellular domain of SymRK is an active kinase with an aspartate-phenylalanine-glycine (DFG)-kinase motif necessary for its function. The kinase function is known to lead to autophosphorylation at the threonine residue in position 593 (Yoshida and Parniske 2005). In contrast, the kinase activity from *Arachis* SymRK was traced back to autophosphorylation of tyrosine 670 (Saha et al. 2016). Probably, SymRK can also phosphorylate other interacting proteins which are described in detail in the next section. When the intracellular domain of SymRK is expressed under a strong promoter, it can lead to spontaneous formation of nodules, similar to the overexpression of full-length SymRK (Ried et al. 2014; Saha et al. 2014). Therefore, most likely all parts of the protein necessary for signal transduction are present in the intracellular domain (Saha et al. 2014).

Interactors of SymRK

As already mentioned, NFR5 has been identified to interact with SymRK in Co-IP experiments (Antolín-Llovera et al. 2014b). This interaction is stronger when the MLD of SymRK is not present. A removal of the whole extracellular domain reduces the interaction though (Antolín-Llovera et al. 2014b). Therefore, the LRR-domain might play a role in the interaction of NFR5 and SymRK. A weak Co-IP signal was also visible when NFR1 was recombinantly expressed in tobacco together with SymRK (Antolín-Llovera et al. 2014b). SymRK might thus form a complex with these two receptors.

A remorin protein called SYMREM1 was reported to interact with SymRK and the LysM-receptors NFR5 and NFR1 as well (Lefebvre et al. 2010). Remorins are known to stabilize membrane nanodomains, which are necessary for the functional signaling of plant receptor-like kinases (Bücherl et al. 2017). Among other functions, SYMREM1 stops NFR1 endocytosis and recruits this receptor to specific nanodomains after ligand binding and is necessary for bacterial infection (Liang et al. 2018).

The mevalonate producing HMGR1 has also been reported to interact with SymRK (Kevei et al. 2007). This is especially interesting because HMGR1 is located in the endoplasmatic reticulum. For a functional interaction *in vivo*, SymRK needs to undergo endocytosis, which is a common part of signal transduction in plant receptors and has been suggested for SymRK as well (Geldner and Robatzek 2008; Dávila-Delgado et al. 2023). Mevalonate is known to play a role for the establishment of RNS and AM (Venkateshwaran et al. 2015).

The exact signaling cascade from SymRK to nuclear calcium spiking is not revealed yet, but a few SymRK interacting proteins give indications to a possible downstream signaling cascade. Yeast-2-hybrid experiments identified two candidates for interaction, which could directly be involved in signal transduction cascades. A Gα-subunit of a tripartite G-protein was reported to interact with and be phosphorylated by soybean SymRK. This Gα-protein could be involved in activating different signaling pathways as a quadruple mutant of this factor exhibited less nodules forming on their roots (Roy Choudhury and Pandey 2022). Another yeast-2-hybrid screen revealed a mitogen activated kinase kinase (MAPKK) called SymRK interacting protein 2 (SIP2) to interact with SymRK. A RNAi knockdown of SIP2 resulted in strongly reduced nodule, primordia and infection thread formation. The exact function of SIP2 is not known yet and SymRK does not seem to phosphorylate the protein (Chen et al. 2012).

Two unconventional receptor interactors were also identified with yeast-2-hybrid experiments: a potential peptide hormone *Glycine max* NORK (*Gm*NORK) interacting secreted peptide 1 (NISP1) is phosphorylated by SymRK and secreted (Fu et al. 2023). Treatment with NISP1 slightly increased nodule formation, whereas knockout of NISP1 resulted in slight reduction of nodule formation (Fu et al. 2023). A second protein called SymRK interacting protein 1 (SIP1) is described to be a transcription factor with an ARID domain binding elements of the *NIN-promoter*. SIP1 is upregulated upon inoculation with compatible symbiotic bacteria (Zhu et al. 2008). This factor could be a shortcut for transcriptional changes upon SymRK activation.

The intracellular domain of SymRK also provides the interaction surface for the cytoplasmic protein Brassinosteroid insensitive 1-Associated receptor Kinase 1 (BAK1) in *L. japonicus* to inhibit its kinase activity. This was reported to be crucial to prevent BAK1 activation upon rhizobial MAMP perception and thus suppressing immunity against Rhizobia (Feng et al. 2021).

Another prominent class of SymRK-interactors are E3-Ubiquitin ligases. E3-ubiquitin ligases represent a very large protein family comprising around 5% of all *A. thaliana* genes. This is not surprising, as ubiquitination is known to play an important role in protein turnover, subcellular distribution and signal transduction (Mazzucotelli et al. 2006). Polyubiquitination with ubiquitin units linked on their lysine residue at position 48 is usually a signal for proteasomal degradation (Siswanto et al. 2018). Other types of ubiquitination e.g., monoubiquitination or polyubiquitination by lysin linkage e.g. at K63 often play a regulatory role in signaling pathways, especially in plant immunity (Zhou and Zeng 2017; Ma et al. 2020).

Among the E3-ubiquitin ligases interacting with SymRK, there is a member of the Seven in absentia (SINA) family, SINA4 (Den Herder et al. 2008, 2012). SINAs are known to have a cysteine-rich RING domain for interaction, two zinc-finger domains and a substrate binding domain (Den Herder et al. 2008; Ong and Solecki 2017; Siswanto et al. 2018). SINA4 is believed to mediate SymRK endocytosis and recycling, as the protein can be detected in dots inside the cell and at cell membrane (Den Herder et al. 2012).

SymRK interacting E3 ligase (SIE3), another SymRK interaction partner identified by yeast-2-hybrid screens, can ubiquitinate SymRK *in vitro* and when overexpressed in tobacco leaves. Knock-down of SIE3 leads to fewer nodules and fewer infection thread formation after inoculation with a compatible symbiont, while overexpression increased the number of infection events (Yuan et al. 2012). A possible interaction with SIP1 identified in yeast-2-hybrid and bimolecular fluorescence complementation may link to a function of SIE3 in the nucleus, where it can be observed as well as in the cytoplasm (Yuan et al. 2012; Feng et al. 2020).

The E3-Ubiquitin ligase plant U-box protein 1 (PUB1) has been identified to interact with SymRK and NFR1, but does not ubiquitinate these receptors. PUB1 instead can be phosphorylated by both of the receptors (Mbengue et al. 2010; Vernié et al. 2016). PUB1 is believed to be a negative regulator of symbiosis, as *pub1* mutants exhibited a higher degree of symbiotic interaction, thus more infection events of AM fungi as well as more nodules after inoculation with compatible Rhizobia, while overexpression of PUB1 leads to a reduced number of symbiotic structures (Mbengue et al. 2010; Vernié et al. 2016).

PUB2, another E3-Ubiquitin ligase, has similar characteristics as PUB1: It interacts with and can get phosphorylated by SymRK and negatively regulates nodulation (Liu et al. 2018). But in contrast to PUB1, PUB2 can ubiquitinate SymRK *in vitro* and when transiently co-expressed in tobacco, a functional PUB2 also leads to proteasomal degradation of SymRK (Liu et al. 2018).

Phylogenetic conservation of common symbiosis genes

Most of the common symbiosis genes described above are highly conserved. Many of them are even conserved in their biochemical properties. This leads to a full complementation of nodulation of respective mutants by genes from distinct plants only capable of arbuscular mycorrhiza (Radhakrishnan et al. 2020). Cyclops from rice or tomato can complement a *L. japonicus cyclops* mutant in RNS and AM. Similarly CCamK from rice and tomato can complement *L. japonicus ccamk* mutant in nodulation and AM (Chen et al. 2007; Yano et al. 2008; Markmann and Parniske 2009). However, for SymRK, the situation is different: when SymRK from rice or tomato is expressed in *L. japonicus* roots, they cannot complement the nodulation phenotype, but the AM phenotype can be restored (Markmann et al. 2008). Surprisingly, SymRK from *Tropaeolum vulgare* (*Tv*) is able to fully complement the *symrk* mutant phenotype of *L. japonicus* (Markmann et al. 2008). This plant belongs to the eurosids and is thus phylogenetically closer to the nodulating FaFaCuRo clade. So, unlike for the other common symbiosis genes, there was a neofunctionalization of SymRK that led to a predisposition of the eurosids for RNS, while maintaining AM symbiosis.

Aims of the Thesis

I aimed for a better understanding of the evolution of receptor-like kinases in the context of a potential predisposition event of RNS with a focus on SymRK. SymRK is especially interesting as it is required for both RNS and AM, however SymRK from non nodulating species can't complement the RNS phenotype of *symrk* mutants of nodulating species such as Lotus. The aim of this thesis was to understand the evolutionary adaption of Lotus SymRK to convey its function in both AM and RNS. I wanted to determine the domain of SymRK which allows nodule formation and bacterial entry.

As a model system I chose SymRK from tomato as a non-nodulting species and compared it to Lotus SymRK. The first aim of the thesis was to confirm, that the Tomato SymRK identified by Markmann and colleagues (2008) in the pre-genomic era is really the homologue of Lotus SymRK.

My next aim was to comprehensively characterize the nodulation and *Rhizobia* infection phenotype of Tomato SymRK complemented Lotus *symrk* mutant roots, especially if bacteria can enter the roots via infection threads and how nodule organogenesis is affected in these roots. To complement these data, I sought to also characterize the arbuscular mycorrhiza phenotype.

To track down the observed differences in complementation capacity, we sought to clarify the contribution of the intracellular domain, transmembrane domain and extracellular domain to the signaling capacity of Lotus SymRK. The next step was to further narrow down the responsible subdomain or amino acid motif in the intracellular domain splitting it up dependent on its conservation status among different nodulating and non-nodulating species. In addition, I intended to clarify, if a difference in ubiquitination patterns between Lotus SymRK and Tomato SymRK could be the reason for different signaling outputs.

As bacterial entry and nodule organogenesis also depends on the Nod-factor-receptor NFR1 and NFR5, my final goal was to find out if the intracellular domain and transmembrane domain of these receptor-like kinase are conserved to complement my functional data on SymRK with insights into the evolution on further bacterial entry receptors.

Results

The previously identified putative Tomato SymRK was confirmed to be the homologue and syntelogue of Lotus SymRK

In a previous study, SymRK homologues from tomato, rice (*Oriza sativa*) and other species were identified by mRNA comparison (Markmann et al. 2008). Since then, many more genomes of other plant species have been published or drastically improved including those of *Solanum lycopersicum* and *Oriza sativa* (Sato et al. 2012; Kawahara et al. 2013). Therefore, I wanted to confirm that the identified Tomato SymRK (*Sl*SymRK) and the *Oriza sativa* SymRK (Rice SymRK, *Os*SymRK) are indeed the homologues to Lotus SymRK (*Lj*SymRK) and *Medicaco truncatula* DMI2 (Medicago DMI2, *Mt*DMI2). To achieve that, I performed a phylogenetic analysis. I searched the most similar protein sequences to the Lotus SymRK protein sequence in the genomes of *S. lycopersicum*, *O. sativa* and *M. truncatula*, a close relative of *L. japonicus* with a well described SymRK homologue called Medicago DMI2 with the help of the BLAST algorithm. I used the first 20 hits to construct a phylogenetic tree using the maximum likelihood algorithm RaxML (Stamatakis 2014). The *L. japonicus* receptor *Lj*RINRK1 was used as an outgroup (Li et al. 2019). In the phylogenetic tree, the described SymRK homologues (*Lj*SymRK, *Mt*DMI2, *Sl*SymRK, *Os*SymRK) formed a clear clade with no other protein (Fig. 1A). This result confirmed the close relationship of the previously identified SymRK versions from rice and tomato and provided evidence, that there were no recent duplications of SymRK in these species.

To further confirm that *Tomato SymRK* is the homologue of *Lotus SymRK*, I did a synteny analysis using the *Medicago DMI2* as a reference, as this genome was already available in the databases of CoGe (https://genomevolution.org/coge/) at time of this study. The analysis identified one syntenic region in *L. japonicus* (CM0177340.r2.m), corresponding to *Lotus SymRK*, with a very high synteny score of 18 validating the approach (Tab. 1, Fig. 1B). For *S. lycopersicum,* two syntelogues were detected (Tab. 1, Fig. 1B): One synthelogue consisted of the gene Solyc02g091590.3.1.1, with a synteny score of 6, that matched the predicted Tomato *SymRK* (Tab.1, Fig. 1B). The second syntelogue (Solyc12g088040.2.3.1) with a score of 4, could not be found in the first 50 BLAST hits, when aligning Lotus SymRK to the Tomato genome (Tab. 1, Fig. 1B). In addition, one syntenic region with a low synteny score of 4 was found in *M. truncatula* itself, which however did not correspond to any identified genes.

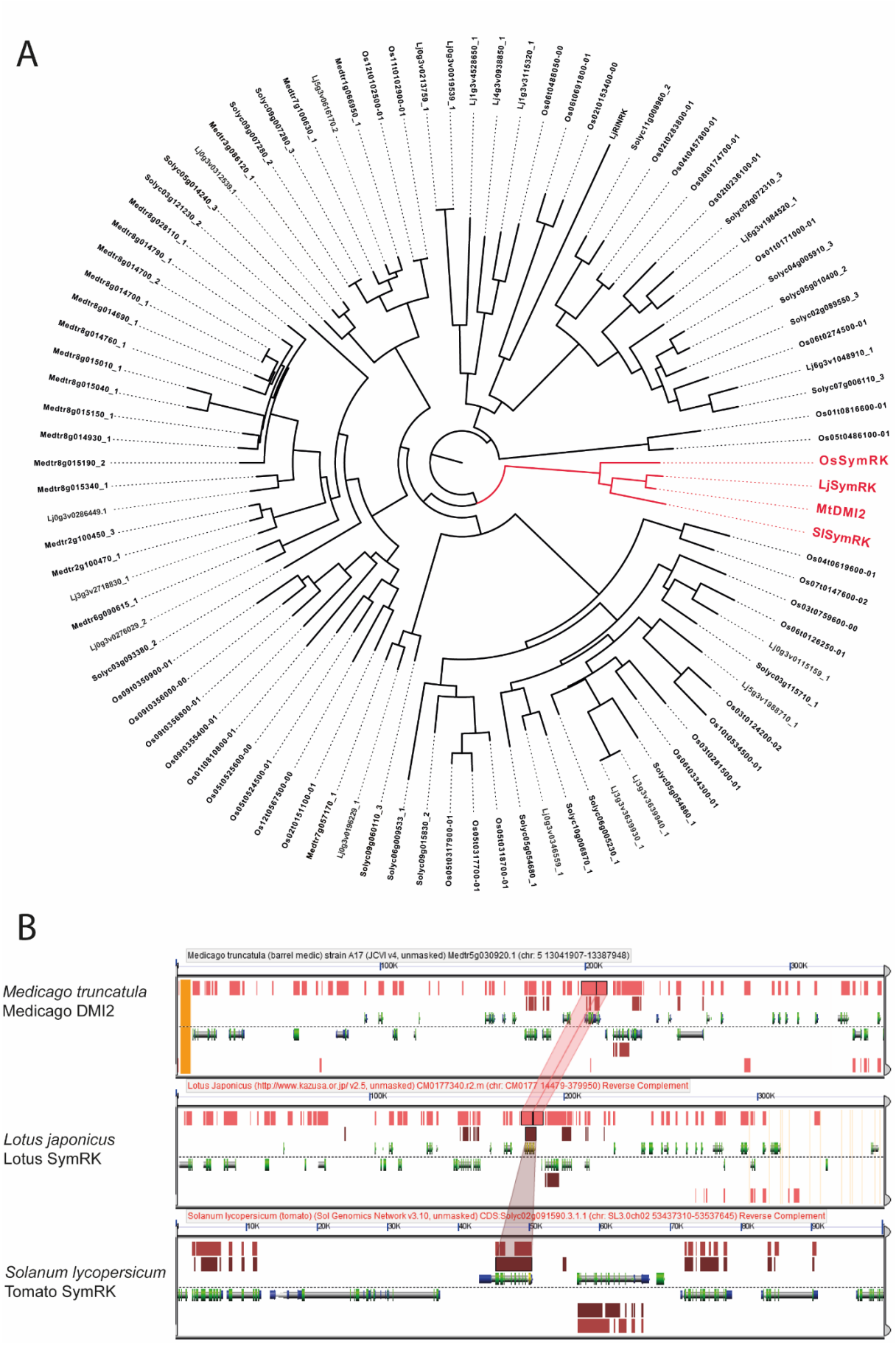
A
OsSymRK
LjSymRK
MtDMI2
SlSymRK
B
Medicago truncatula
Medicago DMI2
Lotus japonicus
Lotus SymRK
Solanum lycopersicum
Tomato SymRK
Medicago truncatula (barrel medic) strain A17 (JCVI v4, unmasked) Medtr5g030920.1 (chr: 5 13041907-13387948)
Lotus Japonicus (http://www.kazusa.or.jp/ v2.5, unmasked) CM0177340.r2.m (chr: CM0177 14479-379950) Reverse Complement
Solanum lycopersicum (tomato) (Sol Genomics Network v3.10, unmasked) CDS:Solyc02g091590.3.1.1 (chr: SL3.0ch02 53437310-53537645) Reverse Complement

Figure 1: Phylogenetic and synteny analysis confirmed the formerly identified Tomato SymRK as the homologue of SymRK in nodulating species. A. For the phylogenetic analysis, Lotus SymRK protein sequence was used in a BLAST search against genomes of *Medicago truncatula (*MtrunA17r5.0*), Solanum lycopersicum* (Heinz SL3.0)*, Oriza sativa* (IRGSP-1.0) and *Lotus japonicus* (MG20 genome v3.0). The first 20 BLAST hits and *Lj*RINRK as an outgroup were aligned, trimmed and a maximum likelihood-tree was established. In the phylogenetic tree, it is visible that Tomato SymRK (*Sl*SymRK), Rice SymRK (*Os*SymRK), Lotus SymRK (*Lj*SymRK) and Medicago DMI2 (*Mt*DMI2) cluster on one branch of the phylogenetic tree, whereas all other proteins are found on different branches, including the outgroup *Lotus japonicus*RINRK. B. For synteny analysis, *Medicago DMI2 (MtDMI2)* was chosen as reference due to the higher genome quality available at the time of analysis. Synteny analysis displays that, the *Medicago DMI2* is syntenic with *Lotus SymRK* and *Tomato SymRK*.

Table 1: Synteny analysis of *MtDMI2* in the genomes of *L. japonicus* and *S. lycopersicum*. Synteny analysis with *MtDMI2* as a reference brings up on syntelogue in the *L. japonicus* genome, two syntelogues in the genome of *S. lycopersicum* and one syntenic region in *M.truncatula* identified by SynFind on https://genomevolution.org/coge/.

Organism	Genome	Type	Name	Chromosome	Synteny score
Medicago truncatula A17	v4 unmasked	query	Medtr5g030920.1	5	0
Lotus japonicus	v2.5 unmasked	syntelogue	CM0177340.r2.m	CM0177	18
Medicago truncatula A17	v4 unmasked	proxy for region	pos 28232678	8	4
Solanum lycopersicum	v3.10 unmasked	syntelogue	CDS:Solyc02g091590.3.1.1	SL3.0ch02	6
Solanum lycopersicum	v3.10 unmasked	syntelogue	CDS:Solyc12g088040.2.1.3	SL3.0ch12	4

Design of domain swap and point mutation constructs to investigate the evolution of SymRK, NFR5 and NFR1

To get a better understanding of the evolution of proteins, chimeric domain swap proteins can be an effective research tool for receptor-like kinases (Miyata et al. 2016; Li et al. 2018). Therefore, Martina Ried and I decided to use this method to get more insights about SymRK functional evolution. We started with swaps of the main known domains of tomato SymRK and Lotus SymRK. These domains are the extracellular domain (ED), divided into the MLD and two or three LRRs, respectively, the transmembrane domain (TM), and the intracellular domain (ID) (Fig. 2A). The amino acid breakpoints of each swap construct used in this study are displayed in supplementary table S1. All constructs were controlled by a native *SymRK* promoter comprising ca. 5000 bp upstream of the SymRK protein coding region. These constructs were used to further elucidate which domain of SymRK evolved for functioning in RNS. For this purpose, we used *Agrobacterium rhizogenes* mediated transformation (“hairy root transformation”) and monitored different conditions and timepoints of symbiotic interactions. We used the *symrk-3* mutant, which has an insertion in Exon 4 of the *SYMRK* gene which consists of 15 Exons ((Perry et al. 2003; Markmann et al. 2008). Based on the results found with these constructs (described below), I continued to narrow down the potential

differences and cloned constructs containing point mutations eliminating potential ubiquitination sites, swapping different parts of the intracellular domain, and put them again under the control of the native SymRK promoter. These constructs were again used in hairy root transformation. Other very important receptors in the establishment of RNS and known interactors of SymRK are *Lj*NFR5 and *Lj*NFR1. Therefore, I subjected the *Lj*NFR5 and *Lj*NFR1 receptors, to the same approach. I cloned a domain swap construct with the extracellular domain of *Lj*NFR5 and the intracellular domain and transmembrane domain of its tomato homologue *Sl*LYK10 and *Sl*LYK10 itself and added the native *LjNFR5* promoter to be used in hairy root transformations. The same was done with *Lj*NFR1 and its tomato homologue *Sl*LYK1: the extracellular domain of *Lj*NFR1 was fused to the intracellular domain and transmembrane domain of *Sl*LYK1. I added the native *LjNFR1*-promoter to *Sl*LYK1 and the swap construct.

Tomato SymRK complemented the AM phenotype of the *symrk-3* mutant, but not the bacterial epidermal infection phenotype

Markmann and colleagues postulated that the variable extracellular domain of SymRK from different species were adapted to their needs in different symbiotic signaling (Markmann et al. 2008). To investigate this hypothesis, my coworker Martina Ried and I performed domain swaps of the full extracellular domain, the transmembrane domain, the intracellular domain, and the MLD, and LRR domain of SymRK from *L. japonicus* and *S. lycopersicum* (Fig. 2A).

I used these constructs, together with an empty vector control, in a hairy root experiment to test the ability of the Lotus SymRK and Tomato SymRK to complement the AM deficient *symrk-3* mutant. I monitored the successful infection rate of the epidermis at 12 days after inoculation with beforehand nursed *R. irregularis* in transformed roots. As described before (Demchenko et al. 2004), the *symrk-3* mutant roots transformed with an empty vector exhibited a high number of hyphae attached to the roots, but very few events of hyphae surpassing the epidermis (Fig. 2B). Roots transformed with either Lotus SymRK or Tomato SymRK displayed a high number of hyphal entry events and only in few cases, the symbiont was attached to the root epidermis without entering it (Fig. 2B). The two most distinct swap constructs, “Lomato” (containing the extracellular domain of Lotus SymRK and the transmembrane and intracellular domain of Tomato SymRK) and “Totus” (with the extracellular domain of Tomato SymRK and transmembrane domain and intracellular domain of Lotus SymRK), were also tested for their complementation capacity in AM symbiosis to confirm their functionality. Roots transformed with these constructs were also able to engage with *R. irregularis* in a similar manner as the roots transformed with Lotus SymRK or Tomato SymRK (Fig. 2B).

Knowing that all constructs could functionally complement the AM phenotype of *symrk-3*, they were also tested for their complementation capacity in root hair infection after inoculation with the bacterial symbiont *Mesorhizobium loti.* I observed the previously described root hair deformation phenotype in *symrk-3* mutant roots transformed with empty vector (Fig. 2C) (Stracke et al. 2002; Miwa et al. 2006). Upon transformation with Lotus SymRK, root hair infection and entrapment formation were observed, to a slightly smaller extent than in the wild type roots transformed with empty vector. A small percentage of root hairs were deformed in wild type and complemented roots as well (Fig. 2C). In contrast to the previous report (Markmann et al. 2008), mutant roots transformed with Tomato SymRK exhibited infection threads and entrapments as well, albeit in very low numbers (Fig. 2C). The majority of root hairs exhibited root hair deformations as observed in mutant roots (Fig. 2C). Surprisingly, the mutant roots transformed with “Totus” reached a comparable level of infection thread and entrapment formations as roots complemented with Lotus SymRK, whereas “Lomato” transformed mutant roots exhibited a pattern similar to Tomato SymRK transformed roots, with a low percentage of infection threads and entrapments and a high number of root hair deformations (Fig. 2C). Thus, the intracellular domain of SymRK seemed to play a more important role for the complementation capacity of SymRK versions than the extracellular domain.

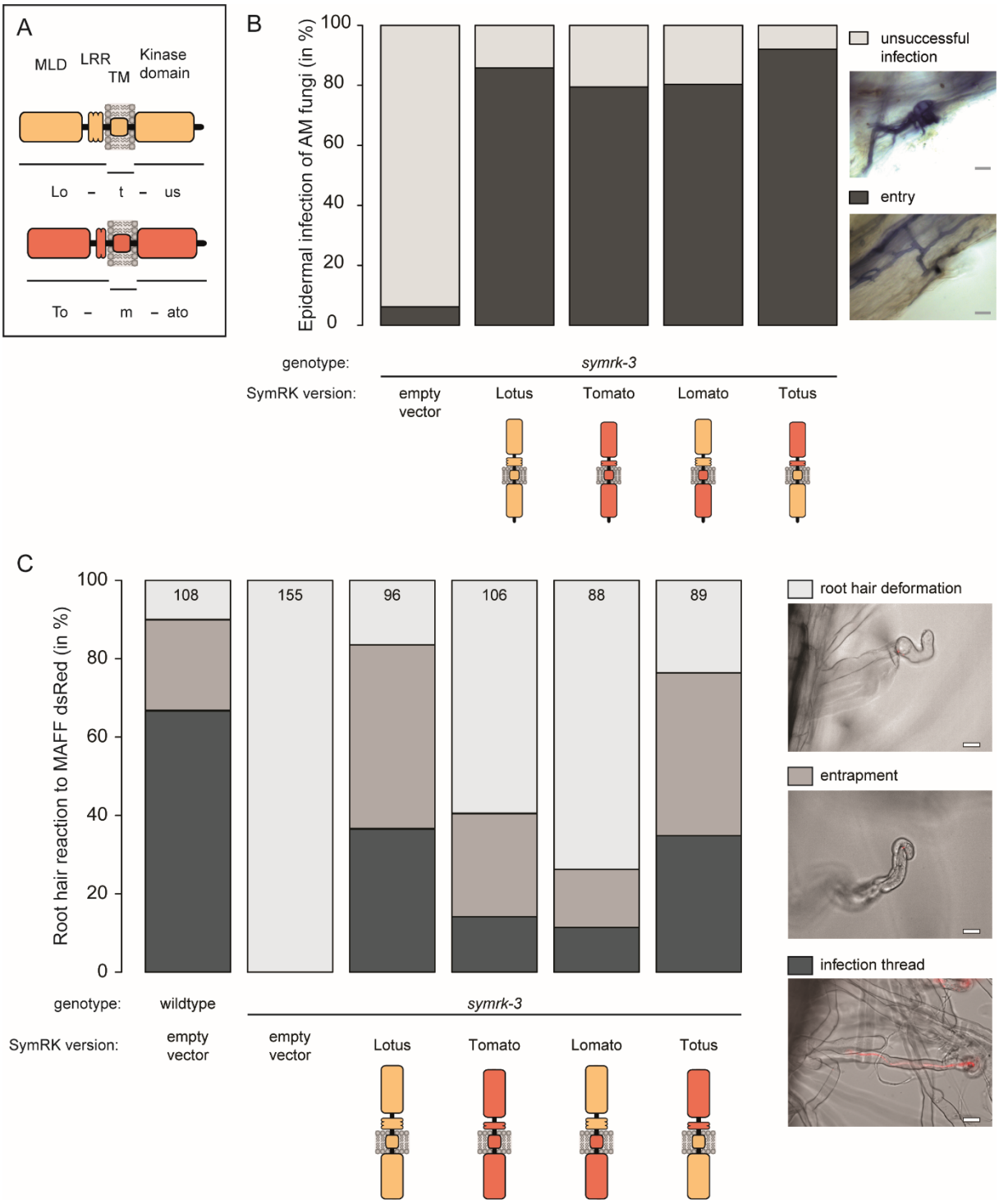

Figure 2: Tomato SymRK and swap constructs containing the intracellular domain of Tomato SymRK can complement the epidermal infection of AM fungi in *Lotus japonicus symrk-3* mutants, but not the epidermal infection with *M. loti* A: Domain structure of Lotus SymRK and Tomato SymRK containing of the extracellular Malectin-like domain (MLD) and Leucin-rich repeats (LRRs), with two LRR-domains in Tomato SymRK and three LRRs in Lotus SymRK. Both have a transmembrane domain and an intracellular kinase domain. The plasma membrane is indicated by the small phospholipid icons. Dashes between the letters of the words Lotus and Tomato indicate the wording of the swap-constructs build for further experiments, with 'Lo' and 'To' standing for the respective extracellular domain, 't' and 'm' indicating the respective transmembrane domain and 'us' and 'ato' indicating the respective intracellular domain. B, C: The *symrk-3* mutant was transformed by hairy root transformation with Lotus SymRK, Tomato SymRK and two swap constructs of these SymRK

versions expressed under the native Lotus SymRK promoter and an empty vector. The wild type was transformed with an empty vector. B: The transformed roots were inoculated for twelve days with *R. irregularis,* that was nursed with chives before. *Symrk-3* mutants transformed with empty vector exhibited low entrance rate of *R. irregularis* as described before, but only attachment to the root surface. *Symrk-3* mutants transformed with Lotus SymRK, Tomato SymRK or one of the swap constructs „Lomato“ or „Totus“, exhibited a high rate of successful epidermal infections. At least 13 plants were scored per genotype. C: The transformed plants were inoculated with *M. loti* MAFF *Ds*Red (indicated by red fluorescence) for seven days. The *symrk-3* mutant transformed with an empty vector did not show any infection threads forming nor entrapments. Instead, the roots displayed many root hair deformations as a reaction to the presence to *M. loti*. In the wildtype, these deformations only appeared at a low abundancy, root hair infections and entrapments were frequent. In roots systems transformed with Lotus SymRK or Totus, the number of root hair deformations was low. The number of entrapments was higher than in the wildtype and the number of infected root hairs was lower. Roots transformed with Tomato SymRK exhibited a very low number of infected root hairs and entrapments, but a high number of root hair deformations. Similar results were observed in those transformed with „Lomato“, which had a slightly lower occurrence of normal infection events (entrapments and infection threads). Root systems transformed with „Totus“, exhibited a low percentage of the root hair deformations. The scale bars represent 20 µm and the numbers in the bar charts represent the total number of inspected root hairs per genotype. At least five plants were inspected per genotype.

Domain swaps confirmed that the intracellular domain of Tomato SymRK cannot fully complement the nodulation phenotype of the *symrk-3* mutant

To further elucidate the importance of the intracellular domain of SymRK in the adaptation to its role in RNS in the rosid clade, Martina Ried and I performed hairy root transformations with many swap constructs as well as truncated versions of SymRK. The *symrk-3* mutant did not exhibit any form of nodule, primordium or another type of cell division, in this work defined as swelling (Fig. 3), when the root systems were exposed to *M. loti* for three weeks. In roots transformed with Tomato SymRK, we observed a strong reduction of nodules compared to those transformed with Lotus SymRK or wildtype roots transformed with empty vector (Fig. 3). This is not in line with the results of Markmann and colleagues, who did not find any nodule formation in roots transformed with Tomato SymRK and inoculated with *M. loti* (Markmann et al., 2008). In addition to the few nodules, excessive swelling formation was observed in Tomato SymRK transformed roots, whereas the number of nodule primordia stayed low (Fig. 3). Unlike nodules (Pierce and Bauer 1983), the swellings were not refined to discrete areas of the root, but expanded throughout the entire root system. We observed the swelling phenotype in roots transformed with all domain swaps containing the intracellular domain of Tomato SymRK (“Totato”, „Lomato“, „Lotato“) (Fig. 3), whereas the roots transformed with domain swaps containing the intracellular domain of Lotus SymRK (“Lomus”, “Tomus”, „Totus“), did not, or only to very low extent exhibit swelling formation (Fig. 3). Strikingly, Lotus SymRK constructs lacking the extracellular domain (LjΔED) or the Malectin-like domain (LjΔMLD) could not complement the mutant phenotype at all (Fig. 3), unlike reported before (Antolín-Llovera et al. 2014b). In contrast, tomato constructs lacking theses domains (SlΔED, SlΔMLD), led to

swelling formation and low nodule and primordia formation as described for Tomato full-length SymRK (Fig. 3).

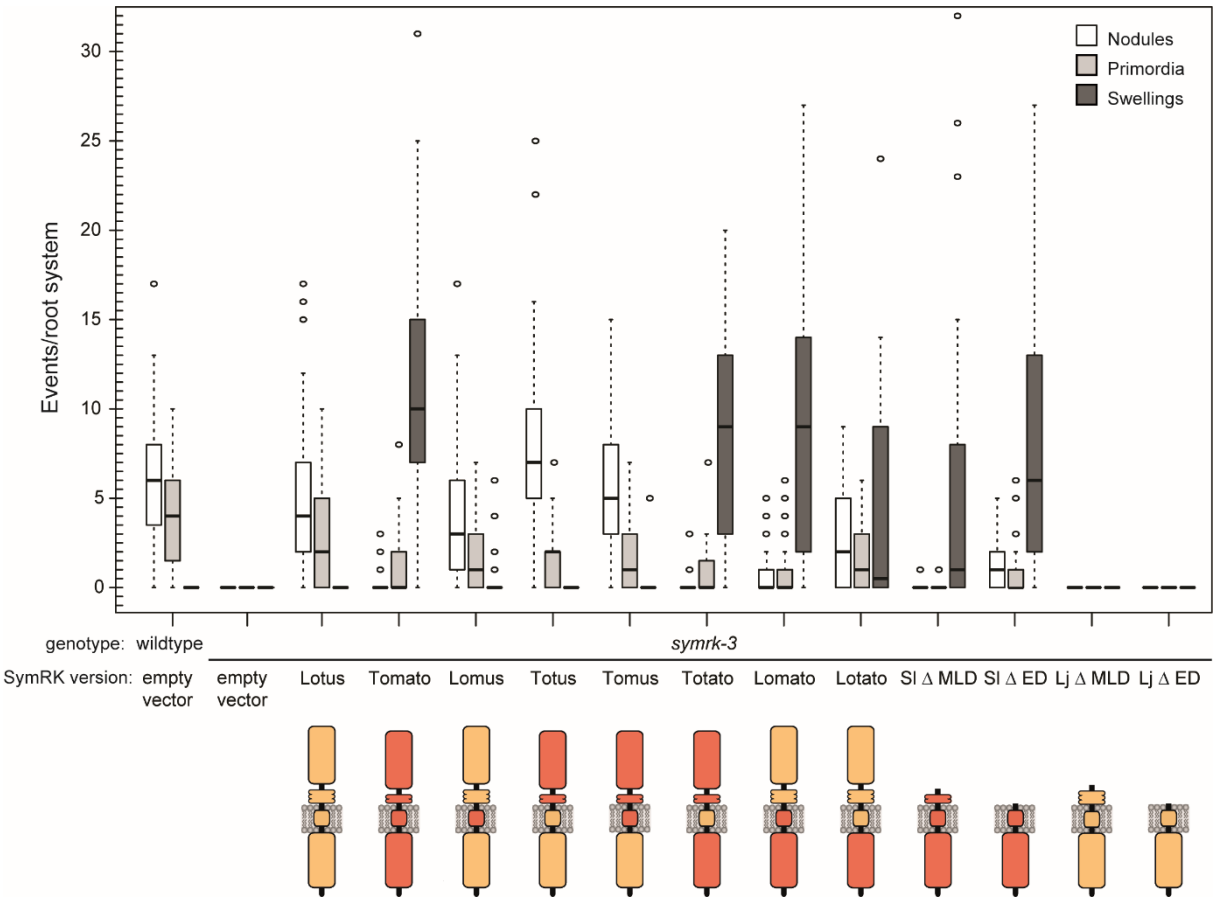

Figure 3: Complementation of *symrk-3* mutant with domain swap constructs containing the intracellular domain of Tomato SymRK showed swellings and reduced nodule formation. The *symrk-3* mutant was transformed with Lotus SymRK, with Tomato SymRK and the respective SymRK swap constructs as described in Fig. 2 under the native SymRK-promoter via hairy root transformation with *A. rhizogenes*. Gifu wildtype plants were transformed with an empty vector and served as a control. The plants were inoculated with *M. loti* MAFF *Ds*Red and analyzed 3 weeks post inoculation. Hairy roots expressing Lotus SymRK showed a comparable number of nodules and primordia as the wildtype. Tomato SymRK transformed roots showed a low number of nodules and primordia, but a high number of abnormally enlarged primordia, called swellings. Swellings only occured in in roots transformed with domain swaps containing the intracellular domain of Tomato SymRK (Totato, „Lomato“, „Lotato“). Constructs containing the intracellular domain of Lotus SymRK show partially a reduced nodule number, but no swelling formation (“Tomus”, „Totus“, “Lomus”). Lotus SymRK constructs lacking the extracellular domain (ED) or the Malectin-like domain (MLD) (LjΔED, LjΔMLD) could not complement the *symrk-3* mutant at all. But the Tomato SymRK without ED or MLD (SlΔED, SlΔMLD) showed nodules, primordia and swellings, comparable to Tomato SymRK transformed roots. The boxplot represents the interquartile range of the data from first to third quartile, the solid line represents the median of the values and the whiskers represent the data range. Outliers as classified by R default settings are represented with open circles. At least 30 plants were scored per genotype. As the data are skewed towards zero values no statistical test was applied.

One construct showed a phenotype that was not in line with the pattern described above: A construct containing the intracellular domain, transmembrane domain and Leucin rich repeats from Lotus SymRK and only the Malectin-like domain from Tomato SymRK did exhibit a high

number of swellings. PCR analysis of the transformed roots suggested the presence of a Tomato SymRK-ID in some of the roots though (data not shown), suggesting a potentially wrong identity of the transformation vector. Therefore, the result of that transformation is excluded from this study, but should be further investigated.

Swellings induced by Tomato SymRK were less structured than primordia and rarely infected

Nodules exhibited wild type like morphology with the bacteria in the center of the nodule and were confined to few areas of the roots, if roots were successfully transformed with Lotus SymRK (Fig. 4). The swellings induced by Tomato SymRK instead appeared in high numbers close to each other all over the root system and did not reveal a clear localization of bacteria (Fig. 4). In histological sections of primordia, the dividing cells formed a roundish shape and cortical infections threads were visible (Fig. 4). Sectioned swellings displayed a broader area of small cells indicating cell division and the cells in the swelling center were less organized (Fig. 4). Bacterial fluorescence was rarely detected inside the swellings but was present in high density on the epidermis (Fig. 4).

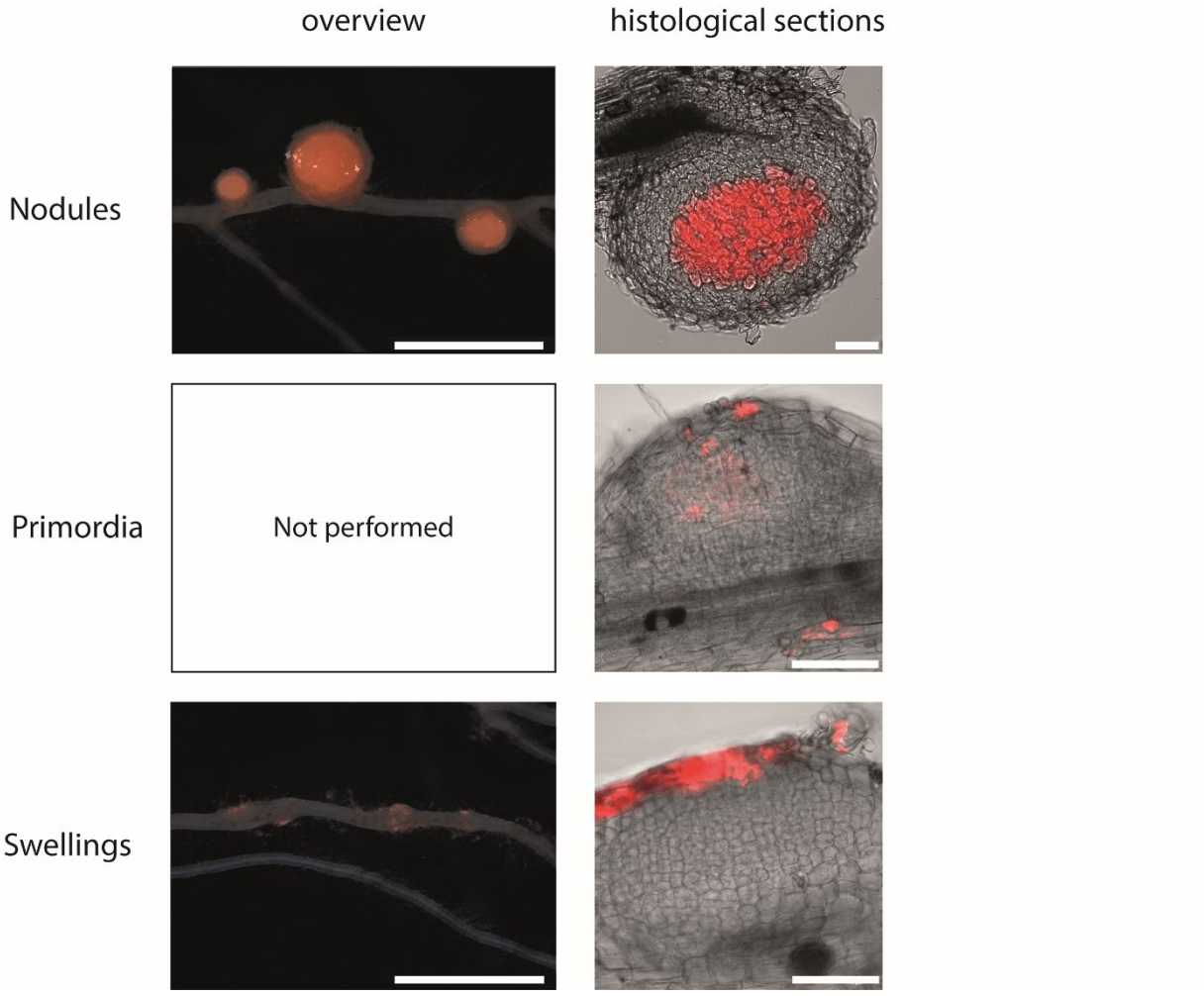

Figure 4: Tomato SymRK dependent swellings are a new phenotype in *L. japonicus* inoculated with *M. loti* MAFF *Ds*Red. Roots expressing Tomato SymRK or Lotus SymRK were analyzed 21 days post inoculation for overview pictures and for histological sections. Red fluorescence and brightfield was merged for overview and histological sections. Roots transformed with Lotus SymRK exhibited high numbers of nodules and primordia. The nodules found in roots transformed with Lotus SymRK were roundly shaped (overview) and inside, a high number of red fluorescing bacteria can be seen, whereas on the outside of the nodules, no bacteria could be detected (histological sections). In the histological sections of a primordium, the cells in the center divided in a pattern that led to a roundish shape of the structure. From the epidermis to the center of the roundly shaped region, bacteria were also visible. On the outside of the primordium, bacteria were also present in low numbers at distinct spots. The overview of swellings exhibited the presence of several swellings close to each other, which is rarely seen with primordia or nodules (data not shown). The cells of the swelling did exhibit the roundish pattern as of the primordium, but seemed to divide in a less ordered fashion (histological section). Inside the swellings, no bacteria were visible, whereas on the outside or epidermal layers of the swellings, the fluorescence signal for bacterial presence was very high. Scale bars: overview: 1.5 mm, histological sections: 100 µm

I was also interested in the fate of swellings after a longer inoculation time with their symbiont. Therefore, I inoculated transformed *symrk-3* mutant roots for 7.5 weeks with *M. loti* MAFF *Ds*Red. In mutant roots transformed with Lotus SymRK, pink nodules with accumulation of leghemoglobin and red fluorescence, indicating rhizobial accommodation could be found as well as primordia, and very rarely white nodules (Fig. 5). When mutant roots were transformed with Tomato SymRK instead, a high number of white nodules without bacterial accommodation, indicated by the lack of red fluorescence, and swellings was observed. In contrast, pink nodules were the exception similar to the number of white nodules found in roots inoculated for three weeks (Fig. 5, Fig. 3). Therefore, I suggest, that most swellings progress into non-infected white nodules.

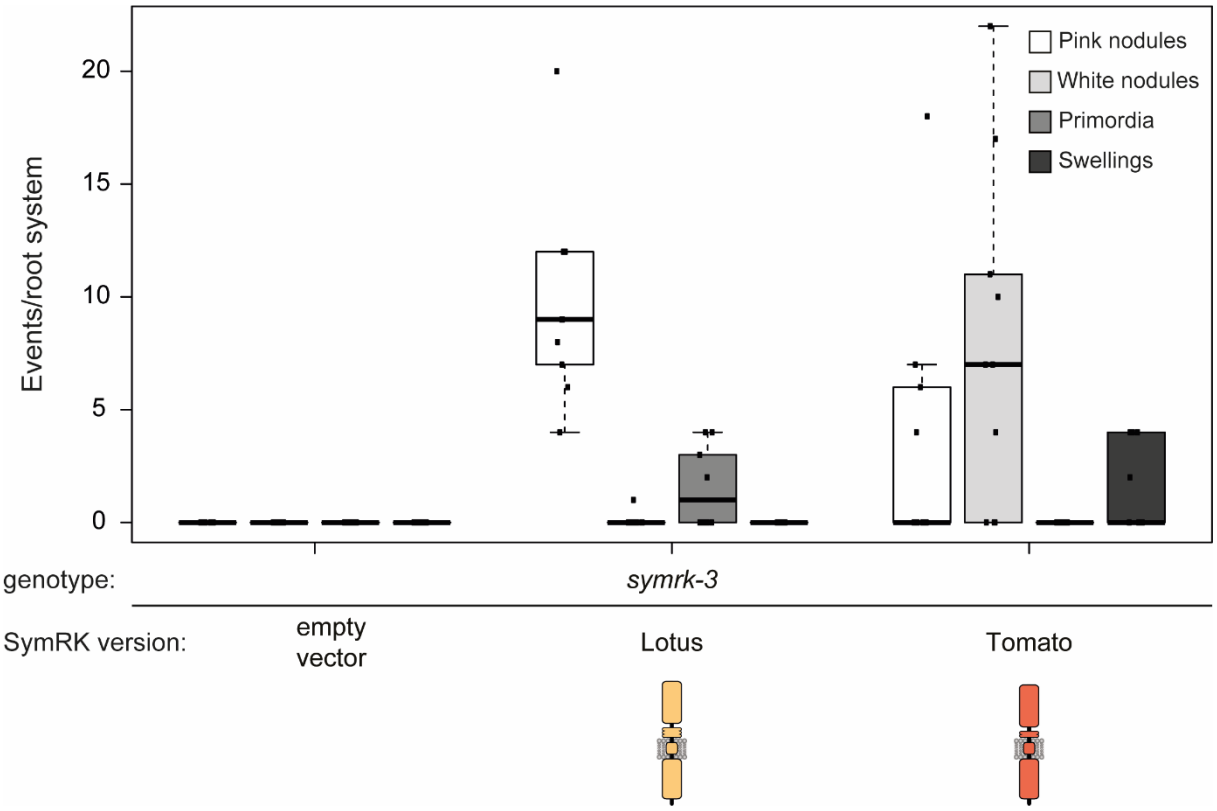

Figure 5: Swellings develop into white nodules when inoculated for 7.5 weeks. The *symrk-3* mutant was transformed via hairy root transformation with an empty vector, Lotus SymRK and Tomato SymRK. The plants were inoculated with *M. loti* MAFF *Ds*Red for 7.5 weeks. In roots transformed with ev, neither nodules, nor primordia, nor swellings could be observed. Roots transformed with Lotus SymRK exhibited a high number of pink nodules, a moderate number of primordia, but nearly no white nodules nor swellings. In root systems transformed with Tomato SymRK, the number of pink nodules was very low except for one root system, instead a high number of white nodules could be observed. No primordia could not be found, but the root systems exhibited a high number of swellings. The boxplot represents the interquartile range of the data from first to third quartile, the solid line represents the median of the values and the whiskers represent the data range excepts outliers as classified by R default settings. All single data points are displayed as closed circles. At least 10 plants were scored per genotype. As the data are skewed towards zero values no statistical test was applied.

The swelling phenotype was consistent in the *symrk-10* mutant

As described above, the rare formation of infection threads, nodules and primordia, and the swelling formation were not reported before in similar experimental set-ups, however with a different *symrk* mutant line (Markmann et al. 2008). In the *symrk-10* mutant, used by the authors of that study, a point mutation in the DFG-kinase motif of SymRK is present, turning it into the kinase-deficient NFG motif (Perry et al. 2003; Markmann et al. 2008). In contrast, the *symrk-3 (cac41.5)* mutant has a 5.8 kb insertion in Exon 4 of the *SymRK* gene (Stracke et al., 2002). In addition, the genetic backgrounds of the mutants might be different, as they have different progenitor lines which were both treated with EMS to induce point mutations (Stracke et al. 2002; Perry et al. 2003). To find out, if the different phenotypes arose because of those differences, I transformed the *symrk-10* mutant with Lotus SymRK, Tomato SymRK, and empty vector as well as the wildtype with an empty vector as a positive control and inoculated the roots with *M. loti*. At 21 days post inoculation, mutant roots transformed with Lotus SymRK and

the wildtype roots displayed a high number of nodules and a lower number of primordia or swellings (Fig. 6). Tomato SymRK transformed mutant roots exhibited a lower number of nodules and a higher number of primordia or swellings (Fig. 6). Overall, the complementation of the *symrk-10* mutant had even a higher degree of complementation compared to the *symrk-3* mutant. This however did not abolish the swelling formation. Taken together I confirmed that the different results between this work and the study of Markmann and colleagues were not caused by the different mutant lines.

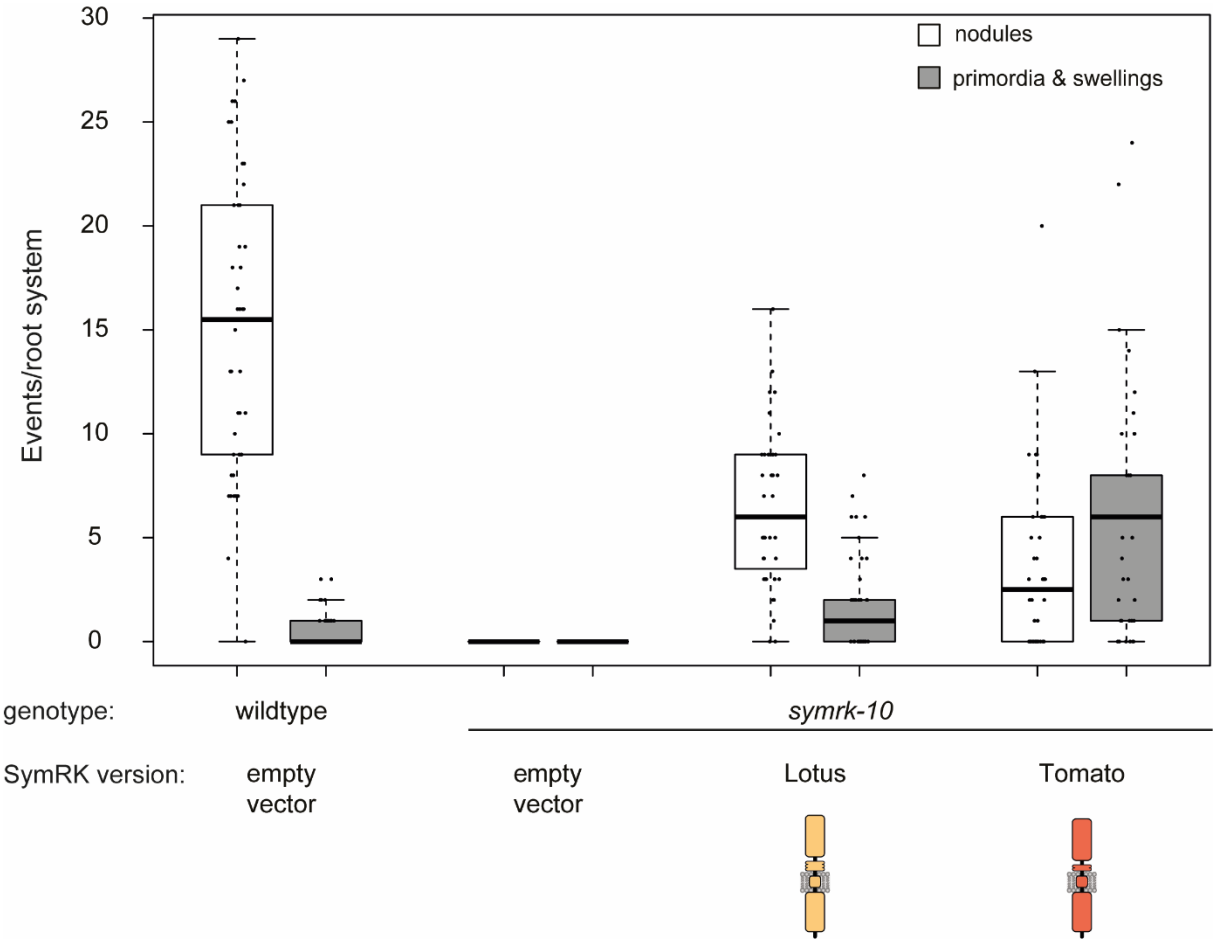

Figure 6: The Tomato SymRK phenotype is consistent in the *symrk-10* mutant. *Symrk-10* mutant plants were transformed via hairy roots with Lotus SymRK and Tomato SymRK. As control, *symrk-10* and the wildtype were transformed with empty vector (ev). The plants were inoculated with *M. loti* MAFF *Ds*Red for 21 days. *Symrk-10* mutant roots transformed with ev did exhibit neither nodule, nor primordia nor swelling formation, whereas in *symrk-10* roots transformed with Lotus SymRK a high number nodules and a moderate number of primordia and swellings could be observed similar to the wildtype. In *symrk-10* mutant roots transformed with Tomato SymRK, the number of nodules observed was lower and the number of primordia and swellings was increased. Compared to *symrk-3* mutant roots transformed with Tomato SymRK, the number of nodules was increased and the number of swellings and primordia reduced. The boxplot represents the interquartile range of the data from first to third quartile, the solid line represents the median of the values and the whiskers represent the data range excepts outliers as classified by R default settings. All single data points are displayed as closed circles. At least 38 plants were scored per genotype. As the data are skewed towards zero values no statistical test was applied.

Differences in ubiquitination sites did not alter the complementation capacity of Lotus SymRK and Tomato SymRK in RNS

The results described above led to the conclusion, that the intracellular domain plays a more important role in the establishment of RNS than the extracellular domain. The intracellular domain of SymRK is also known to be a hub for interactions with many proteins, among them several E3-ubiquitin ligases (Den Herder et al. 2008, 2012; Yuan et al. 2012; Vernié et al. 2016; Liu et al. 2018). Therefore, I hypothesized, that a difference in ubiquitination might cause the impaired function of Tomato SymRK in *L. japonicus* roots in the context of RNS. Consequently, I used the ubpipred algorithm (Radivojac et al. 2010) to predict ubiquitination sites at lysine residues in Lotus SymRK, Medicago DMI2, Tomato SymRK and Rice SymRK. I compared the predicted ubiquitination sites in these SymRK versions concentrating on those either only present in Lotus SymRK and Medicago SymRK and absent in Tomato SymRK and Rice SymRK or vice versa. In addition, I searched for respective lysine residues in an alignment of SymRK intracellular domains of several different species (Fig. 7 - Supplementary Fig. S1). Previous studies reported that SymRK from *Tropaeolum vulgare* was able to complement the *symrk* mutant phenotype, suggesting that a potential differentiation of SymRK enabling the new symbiotic interaction happened at the base of the rosid clade (Markmann et al. 2008). Therefore, the presence of a lysine in the rosids of the alignment, and absence outside this clade or vice versa was the final criterium for ubiquitination candidate selection. There were two sites identified that fulfilled all criteria (Fig. 7A). One predicted ubiquitination site was in the intracellular juxtamembrane domain of the legume SymRKs used for the prediction, that was absent in Tomato and Rice SymRK. In other rosids, lysine residues could be found in close vicinity (Fig. 7A, upper close up). In the C-terminal region, a ubiquitination site was predicted in Rice SymRK, Tomato SymRK, and Maize (*Zea mays*) SymRK, but neither in Lotus SymRK nor Medicago DMI2 (Fig. 7A) and other rosid SymRKs (Fig. 7A, lower close-up).

To elucidate if these ubiquitination sites might have played a role in SymRK sequence evolution, I designed a Lotus SymRK where I exchanged both sites with the respective amino acid from Tomato SymRK (Lotus^{K571MQ915K}), eliminating the predicted ubiquitination site at position 571 and exchanging the respective lysine with the methionine present in Tomato SymRK at the same position based on the alignment and introducing a lysine at position 915 in exchange to a glutamine, where an ubiquitination site was predicted in Tomato SymRK. When I used this construct for hairy root transformation in *symrk-3* mutant roots, they exhibited a slightly increased number of primordia and swellings, compared to mutant roots transformed with Lotus SymRK, but the amount was lower than in roots transformed with Tomato SymRK (Fig. 7 - Supplementary Fig. S2). With this preliminary result, I assumed, single point mutations

at ubiquitination sites might explain parts of the differential signaling capacity of Lotus SymRK and Tomato SymRK. Therefore, I designed and cloned several single point mutation constructs in the Lotus SymRK and the Tomato SymRK background. I eliminated the predicted ubiquitination site in the Lotus SymRK juxtamembrane domain by exchanging it with the methionine present in Tomato SymRK at this position (Fig. 7A, Lotus^{K571M}) and introduced a lysine in the C-terminal tail instead of a glutamine (Lotus^{Q915K}). To mutate the Tomato SymRK, a lysine was introduced in the juxtamembrane domain (Tomato^{M552K}) and the lysine in the C-terminal tail was exchanged by a glutamine (Tomato^{K896Q}). After transforming these constructs in *symrk-3* mutant roots and inoculating them with *M. loti* MAFF *Ds*Red, it was observed that the single point mutations of Lotus SymRK (Lotus^{K571M}, Lotus^{Q915K}) did not display a different phenotype than mutant roots transformed with Lotus wildtype SymRK (Fig. 7B). Roots transformed with Tomato^{M552K} had a phenotype comparable to roots expressing Tomato wildtype SymRK (Fig. 7B). Only the Tomato^{K896Q} construct did not induce any type of nodule, primordia or swelling when introduced to mutant roots (Fig. 7B). To find out if this construct lost its overall capability in symbiotic interactions, I tested its ability to complement the interaction with AM. In mutant roots transformed with Tomato^{K896Q}, I did not observe any AM interaction (Fig. 7 - Supplementary Fig. S3), suggesting an overall non-functional protein rather than a specific impairment in RNS. Altogether, these findings could not provide evidence that single ubiquitination site evolution is the reason for different functions of SymRK in rosids and non-rosids. However, the entire analysis was based on *in silico* predictions of ubiquitination sites and not experimentally verified ubiquitination of SymRK. To this end, no definitive conclusion on the involvement of ubiquitination can be drawn at this stage.

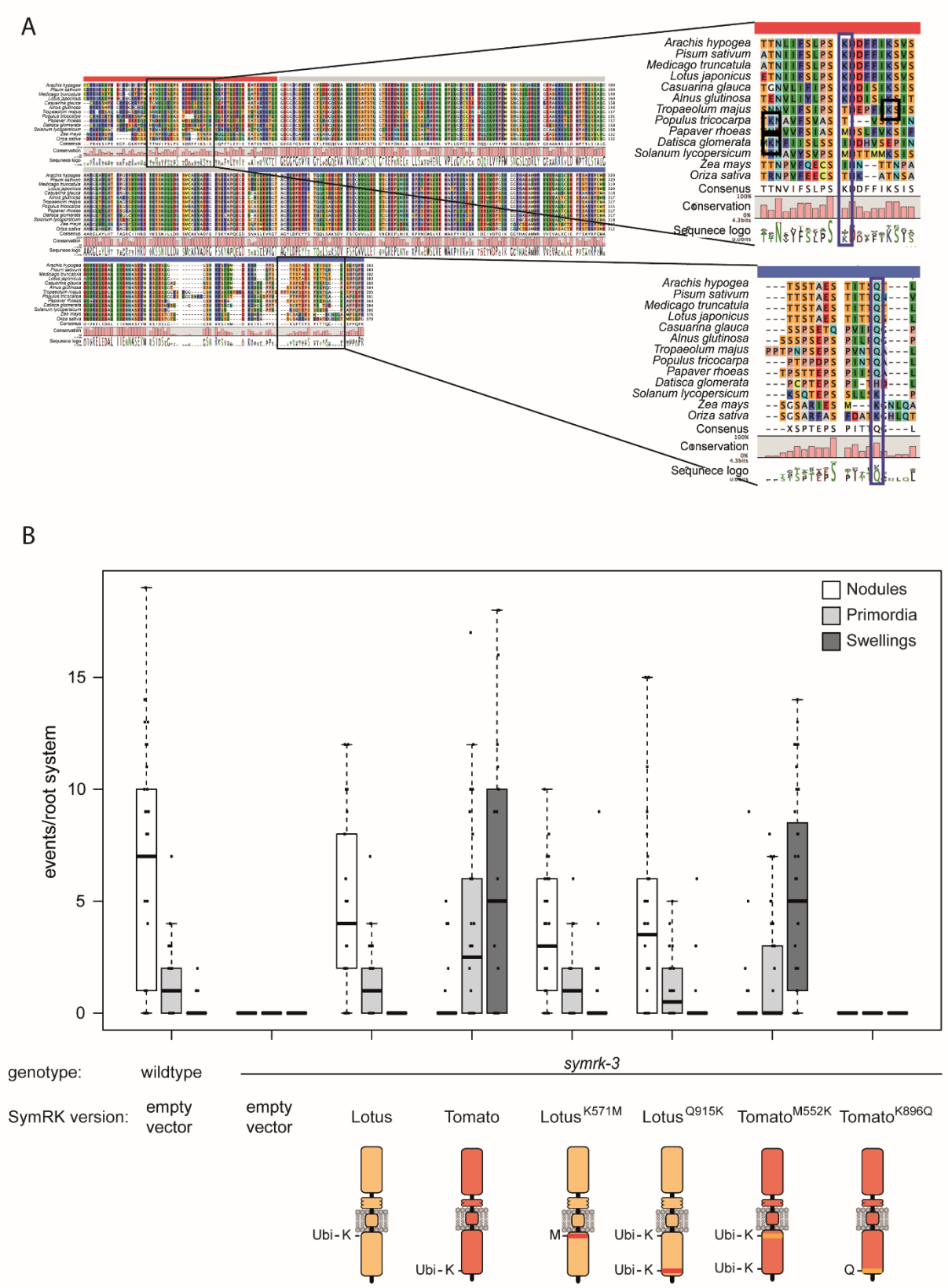

Figure 7: Differences in ubiquitination unlikely play a role for the adaptation of SymRK to function in nodulation. A. Alignment of the intracellular domain of SymRK of rosid (*Arachis hypogea*, *Pisum sativum*, *Medicago truncatula*, *Lotus japonicus*, *Alnus glutinosa*, *Casuarina glauca*, *Datisca glomerata*, *Tropaeolum majus*, *Populus trichocarpa*) and non-rosid (*Papaver rhoeas*, *Solanum lycopersicum*, *Zea mays* and *Oriza sativa*) plant species (For the full alignment see also Fig. 7 - Supplementary Fig. S1). The close-up displays areas, that were identified as potentially ubiquitinated

lysine (K) sites by UbPred prediction software, either in rosid species (upper panel) or in non-rosid (lower panel) only. B. Constructs were designed exchanging the respective amino acids at the identified positions, thus lysine at position 571 of the Lotus SymRK replaced with the methionine present in Tomato SymRK at the respective position (LotusK517M) and vice versa (TomatoM552K) and the glutamine at position 915 of Lotus SymRK was replaced by the potentially ubiquitinated lysine present in Tomato SymRK (LotusQ915K) and vice versa (TomatoK896Q). The *symrk-3* mutant was transformed with empty vector, Lotus SymRK, Tomato SymRK and the respective point mutated SymRK versions. As a control, the wildtype was also transformed with empty vector. After hairy root transformation, the root systems were inoculated with *M.loti* MAFF *Ds*Red for 19 days. The *symrk-3* mutant transformed with empty vector did exhibit neither nodules, nor primordia, nor swellings. The wildtype displayed a high number of nodules, low number of primordia and nearly no swellings. A similar pattern could be observed in mutant roots transformed with Lotus SymRK and both Lotus SymRK ubiquitination variants (LotusK517M and LotusQ915K). Roots containing Tomato SymRK and the Tomato SymRK variant with an additional lysine (TomatoM552K) displayed a high number of swellings and a low number of nodules or primordia. Roots transformed with the Tomato SymRK lacking the lysine in the C-terminus (TomatoK896Q) exhibited no nodules, no primordia and no swellings like the mutant transformed with ev. The boxplot represents the interquartile range of the data from first to third quartile, the solid line represents the median of the values and the whiskers represent the data range excepts outliers as classified by R default settings. All single data points are displayed as closed circles. At least 20 plants were scored per genotype. As the data are skewed towards zero values no statistical test was applied.

All parts of the intracellular domain contribute to complementation capacity

In a new approach to learn more about the functional sequence evolution of the SymRK intracellular domain, I closely inspected the alignment described above (Fig. 7 - Supplementary Fig. S1). I noticed the juxtamembrane (JXT) domain to be very diverse among different species. The amino acid sequence conservation between *L. japonicus* and *S. lycopersicum* in that area was determined as only 38% on protein level (Fig. 7 - Supplementary Fig. S1, Fig. 8). The following domain, containing the DFG kinase motif, is highly conserved among all species with 86% identity between *L. japonicus* and *S. lycopersicum* (KD, Fig. 7 - Supplementary Fig. S1, Fig. 8). The following part of the protein was still relatively conserved, but the amino acid differences increased to a sequence conservation between *L. japonicus* and *S. lycopersicum* of 70% (C-term, Fig. 7 - Supplementary Fig. S1, Fig. 8).

To narrow down the region of the intracellular domain contributing the most to signaling capacity in RNS, I designed and cloned domain swap constructs of these identified regions of the protein. All construct contained a native SymRK promoter to drive gene expression. To avoid introducing any point mutations during the creation of constructs, I identified conserved stretches of amino acids at the borders of the identified regions and used them to create the necessary Golden Gate cloning overhangs. As a control, I also cloned wildtype Tomato SymRK and Lotus SymRK from several fragments with the same approach. In hairy root experiments using the *symrk-3* mutant, both wildtype constructs exhibited the previously observed phenotypes after inoculation with *M. loti* MAFF *Ds*Red, confirming the success of the cloning

strategy (Fig. 3, Fig. 8). Also, the constructs which corresponded to „Lotato“ (Lj-Sl ID) and “Tomus” (Sl-Lj ID) displayed the previously observed pattern: Lj-Sl ID had a high number of swellings, a low number of nodules and few primordia, whereas Sl-Lj ID displayed many nodules, few primordia and nearly no swellings similar to the Lotus SymRK expressing roots (Fig. 3, Fig. 8). The patterns observed in roots transformed with the domain swap mutants were less clear. In domain swaps in the Tomato SymRK background, the proportion of full complementation was substantially lower than in domain swaps in the Lotus SymRK background. The only full complementation observed with a Tomato SymRK extracellular domain and TM domain, in addition to the before described Sl - Lj ID, was found in the Sl – Lj KD – Lj C-term, that had the JXT domain of tomato (Fig. 8). In those with two domains of Lotus SymRK either JXT and C-term (Sl – Lj JXT – Lj C-term) or JXT and KD (Sl – Lj JXT – Lj KD), the phenotype of transformed roots was similar to Sl – Lj ID. If only one domain was derived from Lotus SymRK (Sl – Lj JXT, Sl – Lj KD, Sl – Lj C-term), the number of nodules was drastically reduced and the number of swellings increased, leading to a Tomato SymRK-like phenotype (Fig. 8). When the extracellular domain and transmembrane domain of a construct were from Lotus SymRK, the presence of the Lotus C-term (Lj – Sl JXT – Sl KD) or JXT (Lj – Sl KD – Sl C-term) was sufficient to lead to a full complementation with very low swelling formation. Only the Lotus KD (Lj – Sl JXT – Sl C-term) was not sufficient to reduce swelling formation, but the phenotype remained similar to roots transformed with Lj – Sl ID (Fig. 8). All construct in the Lotus SymRK background containing only one domain of Tomato SymRK (Lj – Sl JXT, Lj – Sl KD, Lj – Sl C-term) displayed very low swelling formation with normal nodule numbers similar to the Lotus SymRK phenotype (Fig. 8).

The results of the intracellular domain swaps implicated, that neither a specific domain alone is sufficient for complementation of RNS, nor any domain is absolutely necessary. In addition, it suggested that the extracellular domain or transmembrane domain play a more important role than previous results indicated (Fig. 3).

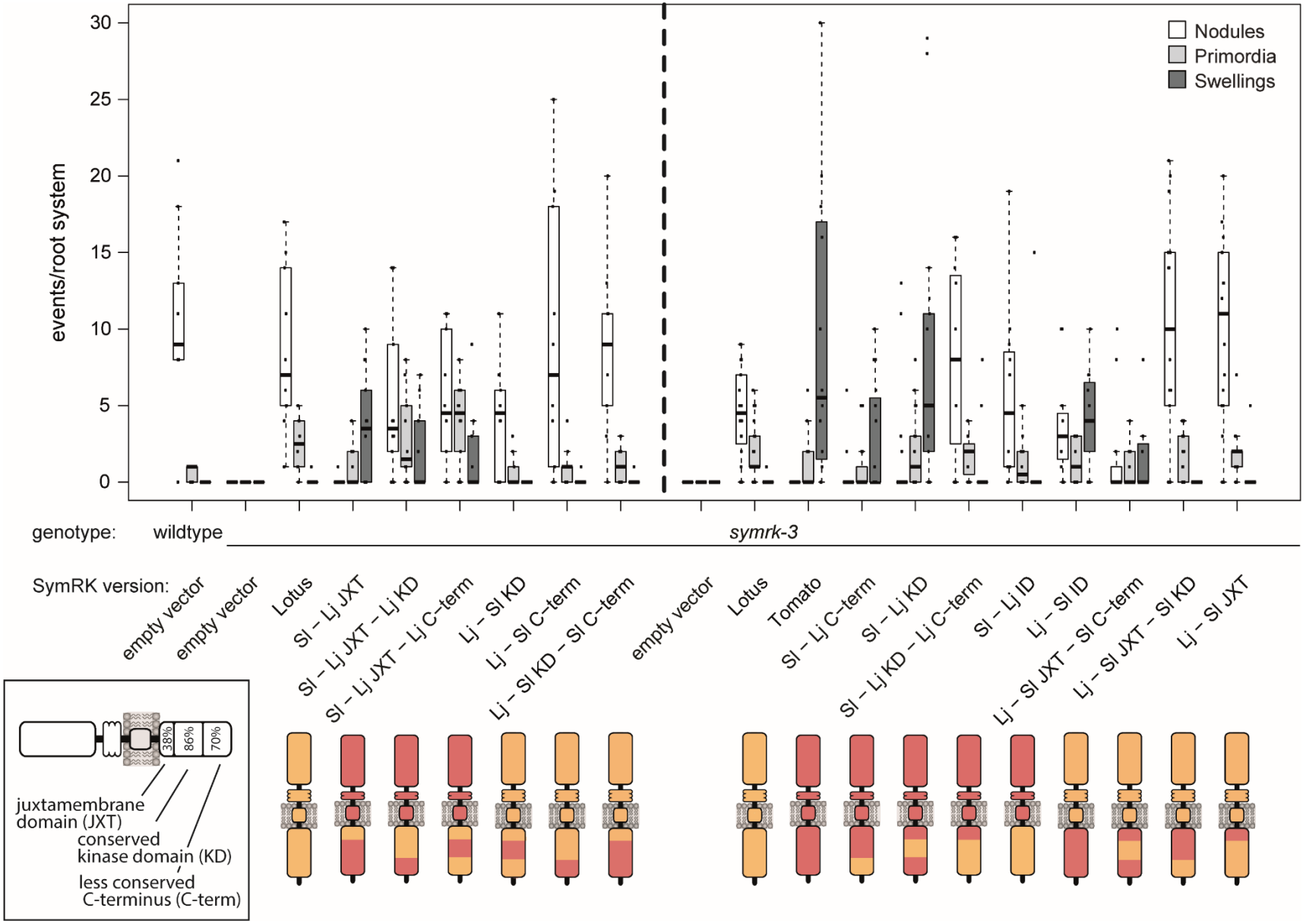
events/root system
30
25
20
15
10
5
0
Nodules
Primordia
Swellings
genotype:
wildtype
symrk-3
SymRK version:
empty vector
empty vector
Lotus
Sl – Lj JXT
Sl – Lj JXT – Lj KD
Sl – Lj JXT – Lj C–term
Lj – Sl KD
Lj – Sl C–term
Lj – Sl KD – Sl C–term
empty vector
Lotus
Tomato
Sl – Lj C–term
Sl – Lj KD
Sl – Lj KD – Lj C–term
Sl – Lj ID
Lj – Sl ID
Lj – Sl JXT – Sl C–term
Lj – Sl JXT – Sl KD
Lj – Sl JXT
38%
86%
70%
juxtamembrane domain (JXT)
conserved kinase domain (KD)
less conserved C-terminus (C-term)

Figure 8: All parts of the Lotus SymRK intracellular domain contribute to nodulation ability. Alignments of the intracellular domains Tomato and Lotus SymRK showed a sequence similarity of 38% in the juxtamembrane domain (JXT), 86% in the conserved part of the kinase domain (KD) and 70% in the C-terminal domain (C-term) (Fig. 7 – supplementary Fig. S1). The *symrk-3* mutant was transformed with empty vector, Lotus SymRK, Tomato SymRK and 13 constructs swapping the juxtamembrane domain (JXT), the conserved kinase domain (KD) and less conserved C-terminus (C-term) in two independent experiments indicated by the dashed line. As a control, wildtype plants were transformed with an empty vector. After hairy root transformation, the root systems were inoculated with *M.loti* MAFF expressing *Ds*Red for 21 or 19 days, respectively. Wildtype roots exhibited a high number of nodules, a moderate number of primordia and no swellings in both experiments, whereas the *symrk-3* mutant roots transformed with empty vector did neither exhibit nodules, nor primordia, nor swellings. Mutant roots containing Lotus SymRK (in both experimental set-ups) as well as Sl – Lj ID ("Tomus") exhibited nodule and primordia formation and very low swelling formation as observed previously (Fig. 3). A similar phenotype presented in roots transformed with constructs in the Lotus SymRK background with only one domain from Tomato SymRK (Lj – Sl KD, Lj – Sl JXT, Lj – Sl C-term), the constructs in the Lotus SymRK background with the Tomato KD and either Tomato JXT or Tomato C-term (Lj – Sl JXT – Sl KD, Lj – Sl KD – Sl C-term) and the construct in the Tomato background containing the Lotus KD and C-term (Sl – Lj KD – Sl C-term). Roots transformed with Tomato SymRK exhibited high numbers of swellings and a low number nodules or primordia as described before (Fig. 3). When only one of the parts of intracellular domain was swapped with Lotus SymRK version (Sl – Ll JXT, Sl – Lj KD, Sl – Lj C-term), a similar phenotype was observed. As described before (Fig. 3), roots transformed with a construct containing the extracellular domain and transmembrane domain of Lotus SymRK and the intracellular domain of Tomato SymRK (Lj – Sl ID/"Lotato") exhibited nodule formation lower than in roots transformed with Lotus SymRK but higher than in those transformed with Tomato SymRK, and swelling formation lower than in Tomato SymRK transformed roots. In roots transformed with constructs in the Tomato SymRK background with the Lotus JXT and the Lotus C-term or the Lotus KD (Sl – Lj JXT – Lj KD, Sl – Lj JXT – Lj C-term) a similar phenotype was observed. With an overall lower number of events, the roots transformed with a construct in the Lotus SymRK background containing the JXT and the C-term of Tomato SymRK (Lj – Sl JXT – Sl C-term) exhibited a similar pattern. The boxplot represents the interquartile range of the data from first to third quartile, the solid line represents the median of the values and the whiskers represent the data range excepts outliers as classified by R default settings. All single data points are displayed as closed circles. As the data are skewed towards zero values no statistical test was applied.

The intracellular domain of NFR5, a known interaction partner of SymRK, is functionally conserved in tomato

Previous results suggested that, besides the crucial contribution of the intracellular domain for SymRK function, the extracellular domain might play a substantial role as well. Lotus NFR5 (*Lj*NFR5) is a known interaction partner of SymRK containing an extracellular domain, a transmembrane domain and an intracellular domain (Antolín-Llovera et al. 2014b; Ried et al. 2014). In tomato, Tomato LYK10 (*Sl*LYK10) was identified as a homologue of NFR5, that plays a role in AM formation (Buendia et al. 2016). Therefore, I created a swap construct with the extracellular domain of *Lj*NFR5 and the transmembrane and intracellular domain of *Sl*LYK10 (*Lj*NFR5ED-*Sl*LYK10TMID). This construct, together with wildtype *Sl*LYK10 and *Lj*NFR5, all under the control of the *Lj*NFR5 promoter, were used for hairy root transformation in the *L. japonicus nfr5-2* mutant background. As a control, the mutant as well as the wildtype were also

transformed with empty vector constructs. At 21 days post inoculation with *M. loti* MAFF *Ds*Red, the wildtype had a high number of nodules and few primordia (Fig. 9A). The mutant roots transformed with empty vector did form neither any nodule, nor primordia. The same was observed in mutant roots complemented with the *Sl*LYK10 construct. Mutant roots transformed with *Lj*NFR5 or the swap construct had a wildtype-like phenotype with a high number of nodules and few primordia (Fig. 9A) in line with a previous report (Seidler 2017). In microscopic observation, the distribution and shape of the nodules formed in roots expressing the swap construct were also comparable to those observed in roots complemented with *Lj*NFR5 (Fig. 9B). This implicated, that the intracellular and transmembrane domain of *Lj*NFR5 and *Sl*LYK10 are functionally conserved. To elucidate the role of *Sl*LYK10 further, I prepared overexpression constructs where *Lj*NFR5 and *Sl*LYK10 were expressed under the control of the strong *L. japonicus* ubiquitin promoter (Maekawa et al. 2008). I confirmed that overexpression of the receptor-like kinases *Lj*NFR5 and *Lj*SymRK leads to the formation of spontaneous nodules without any symbionts (Ried et al. 2014). In contrast, the overexpression of *Sl*LYK10 did not trigger the formation of any spontaneous nodules (Figure 10). This suggested, that a higher receptor abundancy alone is not sufficient to overcome the differences between *Lj*NFR5 and *Sl*LYK10.

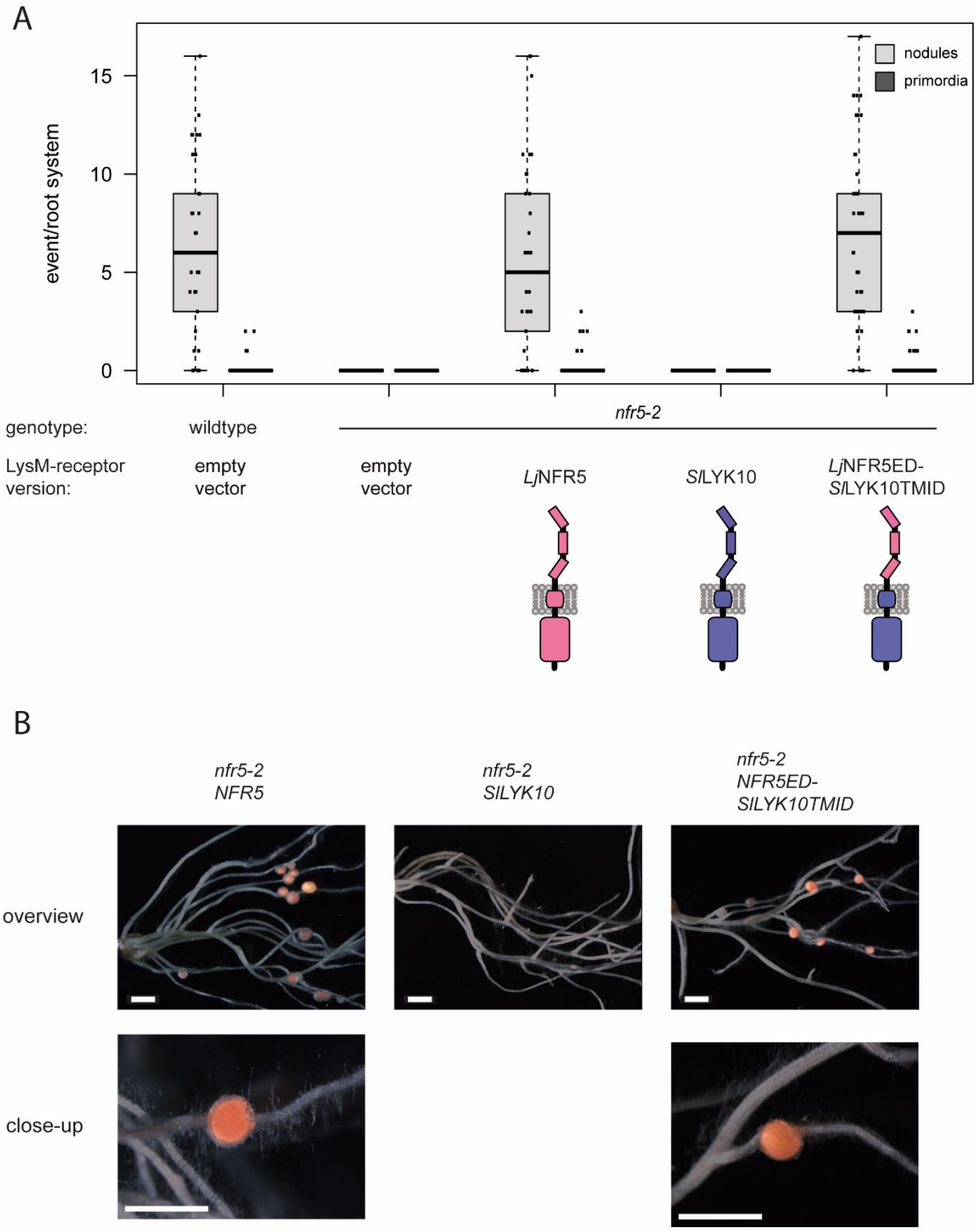

Figure 9: The intracellular domains of *Lj*NFR5 and its tomato homologue *Sl*LYK10 are functionally conserved. *Lj*NFR5, *Sl*LYK10 and a swap construct containing the intracellular and transmembrane domains of *Sl*LYK10 and the extracellular domain of *Lj*NFR5 (LjNFR5ED-*Sl*LYK10TMID) with a native promoter were used for hairy root transformation in the *nfr5-2* mutant. As a control mutant and wildtype plants were transformed with empty vector. Plants were inoculated with *M. loti* MAFF *Ds*Red for 21 days. A. Wildtype plants displayed a high number of nodules and a low number of primordia. Mutant plants transformed with empty vector did neither show any nodule nor primordium formation. Upon transformation with *Lj*NFR5 and NFR5ED, mutant roots exhibited similar number of nodules and primordia as observed in wildtype root systems. In mutant roots transformed

with *Sl*LYK10, neither nodules nor primordia could be observed. B. Root systems transformed with NFR5ED- *Sl*LYK10TMID exhibited nodules of a normal distribution (overview, upper panels) and shape (close-up, lower panels) as mutant roots transformed with *Lj*NFR5. Root systems transformed with *Sl*LYK10 did neither display any nodules nor primordia (overview). Scale bars: 1.5 mm. The boxplot represents the interquartile range of the data from first to third quartile, the solid line represents the median of the values and the whiskers represent the data range excepts outliers as classified by R default settings. All single data points are displayed as closed circles. At least 33 plants were scored per genotype. As the data are skewed towards zero values no statistical test was applied.

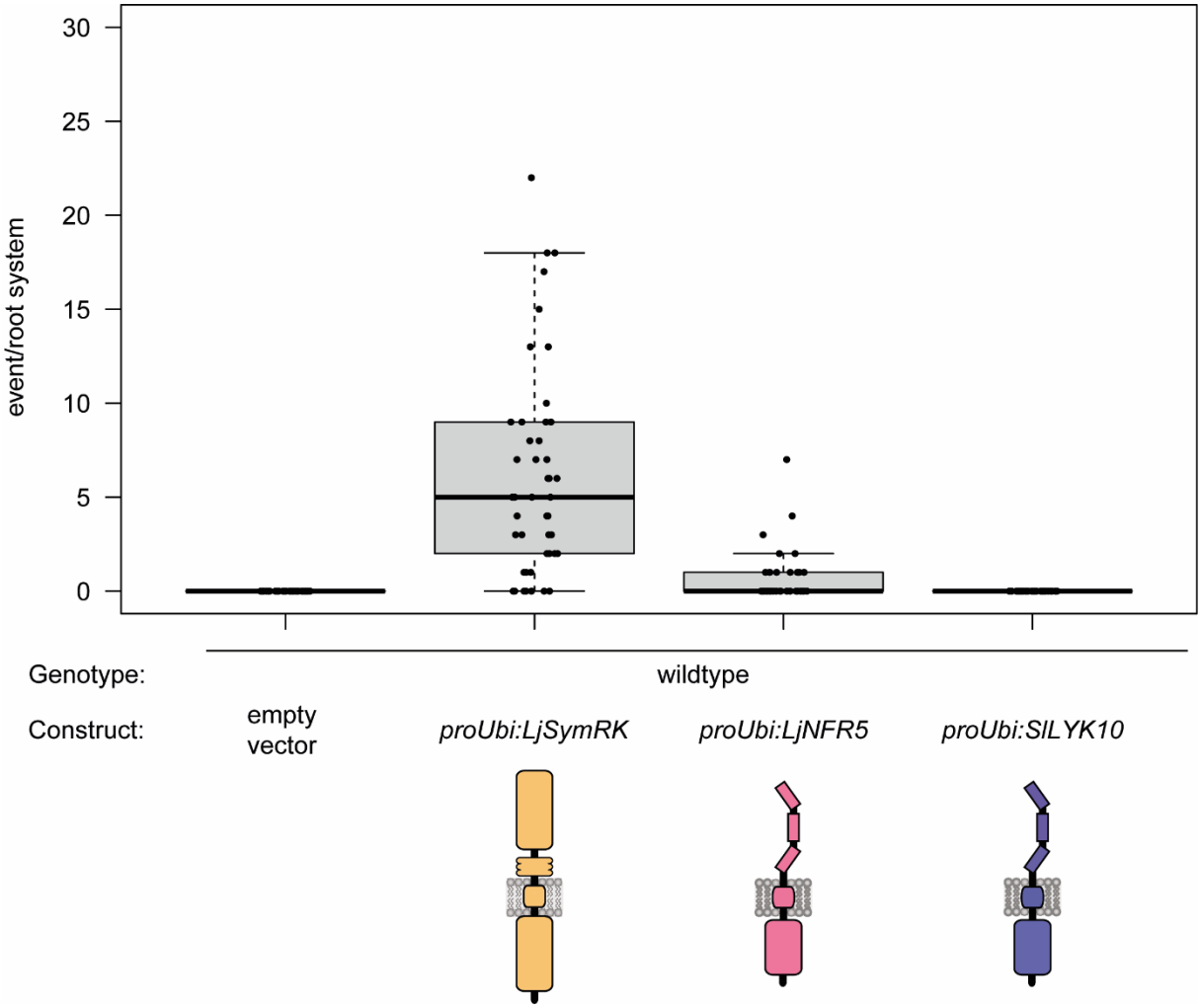

Figure 10: Overexpression of *Sl*LYK10 does not lead to spontaneous nodule formation in contrast to overexpression of *Lj*NFR5 and *Lj*SymRK. Wildtype roots were transformed viahairy roots with an empty vector, *Lj*SymRK, *Lj*NFR5 and *Sl*LYK10 under the control of the *L. japonicus* ubiquitin promoter, leading to overexpression of the genes. Note that overexpression of *Lj*SymRK leads to a higher number of spontaneous nodules and a higher frequency of nodulating plants than overexpression of *Lj*NFR5. The overexpression of *Sl*LYK10 did not lead to the formation of any spontaneous nodules. The boxplot represents the interquartile range of the data from first to third quartile, the solid line represents the median of the values and the whiskers represent the data range excepts outliers as classified by R default settings. All single data points are displayed as closed circles. At least 40 plants were scored per genotype. As the data are skewed towards zero values no statistical test was applied.

The intracellular domain function of NFR1, the co-receptor of NFR5, is partially conserved in tomato as well

*Sl*LYK1 was postulated to be the closest homologue of *Lj*NFR1 and *Os*CERK1, a bifunctional receptor involved in immunity and AM symbiosis in rice (Miyata et al. 2014; Liao et al. 2018; Yang et al. 2022). The kinase domain of *Lj*NFR1 and other closely related RLKs in *L. japonicus* like *Lj*LYS6 or *Lj*LYS7 have a conserved YAQ motif in their kinase domain missing in the *A. thaliana* homologue *At*CERK1 and seemingly necessary for RNS signaling (Nakagawa et al. 2011). Based on this finding, Miyata et al. (2014) showed, that the kinase domains of putative NFR1/CERK1 homologues with the YAQ motif from several species can partially take over the *Lj*NFR1 function in RNS in *L. japonicus*. For a swap construct with the kinase domain of the tomato homologue, a reduced complementation capacity compered to native *Lj*NFR1 was described (Miyata et al. 2014). It is suggested, that the not only the kinase domain, but also the transmembrane and other parts of the intracellular domain play a role in interactions with other RLKs like SymRK (Antolín-Llovera et al. 2014a). Therefore, I tested the RNS complementation capacity of a swap construct of the transmembrane and intracellular domain of *Sl*LYK1 together with the extracellular domain of *Lj*NFR1expressed under the control of the *LjNFR1*-promoter (ca. 2 kb upstream of the *LjNFR1* gene). In a hairy root complementation assay of the *nfr1-1* mutant, the *Lj*NFR1 expressing root systems exhibited nodule formation as expected, even though only 18 out of 45 root systems did respond with nodule formation to the symbiont. Root systems transformed with *Sl*LYK1 did neither exhibit any nodule, nor primordia formation. The swap construct expressing roots were able to form nodules, but in a lower number than the root systems transformed with *Lj*NFR1 (Fig. 11). In addition, even fewer root systems responded to the symbiont (11/42). This suggests, that the intracellular and transmembrane domain of *Lj*NFR1 and *Sl*LYK1 are partially conserved, but *Sl*LYK1 cannot fully restore the function of *Lj*NFR1 in the context of RNS.

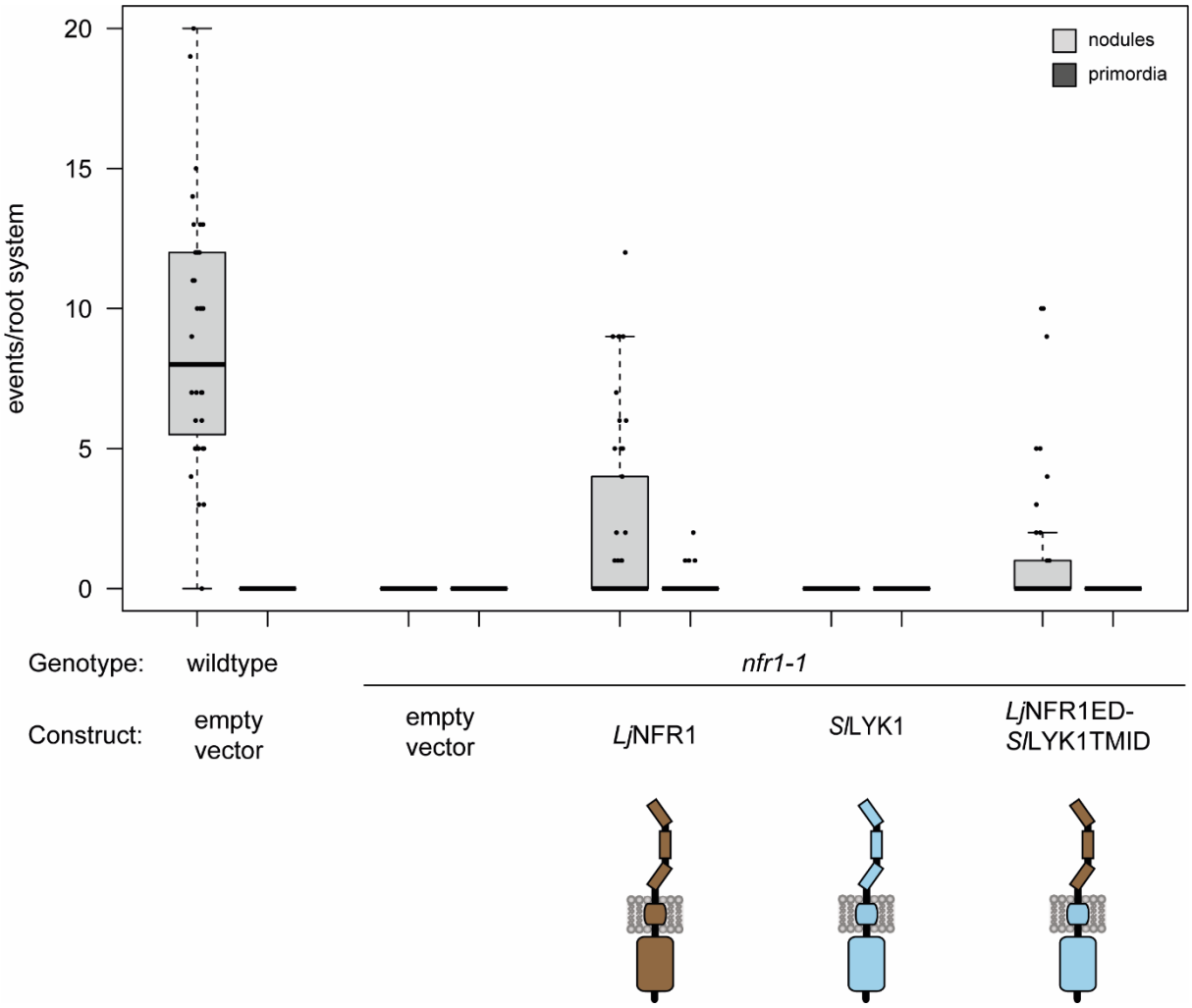

Figure 11: A swap construct of the extracellular domain of *Lj*NFR1 and the intracellular domain and transmembrane domain of *Sl*LYK1 can partially complement the *nfr1-1* mutant. The *nfr1-1* mutant was transformed via hairy roots with *Lj*NFR1, *Sl*LYK1 and a swap construct consisting of the extracellular domain of *Lj*NFR1 and the transmembrane domain and the intracellular domain of *Sl*LYK1 (*Lj*NFR1ED-*Sl*LYK1TMID) under the control of the *Lj*NFR1-promoter and an empty vector control. The wildtype was transformed with an empty vector. The plants were subjected to their symbiont *M. loti* MAFF *Ds*Red for 21 days. The wildtype exhibited a high number of formed nodules, whereas *nfr1-1* root systems transformed with empty vector did not form any nodules. *Nfr1-1* mutant root systems expressing *Lj*NFR1 did exhibit nodule formation, but to a lower number than the wildtype root systems. Neither nodules nor primordia were formed on *nfr1-1* mutant root systems expressing *Sl*LYK1. The number of nodules was low when *nfr1-1* mutant roots expressed *Lj*NFR1ED-*Sl*LYK1TMID, but nodulation was generally enabled by this construct. The boxplot represents the interquartile range of the data from first to third quartile, the solid line represents the median of the values and the whiskers represent the data range excepts outliers as classified by R default settings. All single data points are displayed as closed circles. At least 35 plants were scored per genotype. As the data are skewed towards zero values no statistical test was applied.

Discussion

Tomato SymRK can partially take over symbiotic functions in *L. japonicus*

SymRK has been described as a major component of the common symbiosis pathway (Endre et al. 2002; Stracke et al. 2002; Kistner et al. 2005). Unlike other important common symbiosis genes such as *Cyclops* and *CCaMK* (Banba et al. 2008; Yano et al. 2008), *SymRK* is not functionally conserved in RNS when phylogenetically distant versions of the gene were transferred in respective mutants of *L. japonicus* or *M. truncatula* (Markmann et al. 2008). Markmann and colleagues described no sign of interaction with compatible symbiotic bacteria when the *symrk-10* mutant was complemented with several SymRK versions of species outside the Eurosid clade, including Tomato SymRK and Rice SymRK. In contrast, the arbuscular mycorrhiza phenotype was fully restored with all SymRK versions. Interestingly, *Tv*SymRK could restore AM as well as RNS in the mutant (Markmann et al. 2008). However, in our experiments, roots transformed with Tomato SymRK were able to partially restore nodulation in roots of *symrk-3* and *symrk-10* mutants. Interestingly, few plants exhibited a wild type-like number of nodules and nodule primordia, whereas other plants did develop few to no round-shaped, pink nodules but instead a high number of primordia-like structures that we called swellings.

This observation was also reflected in the root hair response: I observed few shepherd's crook-like infection threads and entrapments, but a high percentage of root hairs could not form entrapments, but instead exhibited deformations. The formation of swellings and root hair responses implies that the presence of Tomato SymRK allows a signaling cascade to the nucleus to some extent, but not sufficient for successful bacterial accommodation. The *symrk-14* mutant, with a mutation in the glycin-aspartate-prolin-cystein (GDPC) domain of the MLD, leads to a similar phenotype with abundant primordia, but rare nodule formation (Kosuta et al. 2011). The root hair phenotype described for this mutant (Kosuta et al. 2011), also resembles the phenotype observed in roots expressing Tomato SymRK after inoculation with compatible bacteria. This suggests that certain perturbations in SymRK signaling either by sequence mutation or trans-species complementation lead to a similar phenotype.

Possibly, the synthesis of slightly corrupted SymRK versions results in problems in the protein folding or trafficking of *L. japonicus*. For example, when the human cystic fibrosis transmembrane conductance regulator (CFTR) has a deletion of one amino acid (Δ508F), it is still a functional channel, but does not make its way to the plasma membrane. The truncated protein is recognized by chaperones to fold to slowly, is not released from the endoplasmatic reticulum, and degraded by the ubiquitin-proteasome pathway (Cheng et al. 1990; Pind et al. 1994; Amaral 2004). A similar mechanism might lead to a non-correct number of SymRK version in the plasma membrane and therefore a disrupted signaling cascade. An uncontrolled protein number at the plasma membrane might also explain why in some plants by chance

epidermal infection and nodule formation can happen. *A. rhizogenes* mediated insertions in the host genome happen randomly and often more than one copy is inserted into the genome (Collier et al. 2005). The genomic environment of the insertion and the number of transformation events might influence the expression strength from nearly no expression to a high, or ubiquitous expression in the respective root systems. A high presence of sub-functional Tomato SymRK protein at the plasma membrane might be sufficient to restore the full, functional signaling cascade. Along this line the entire nodule formation process can be triggered by overexpression of symbiotic receptor-like kinases (Ried et al. 2014).

Additionally, it is possible that the role of SymRK in RNS is more defined and spatiotemporal restricted than previously hypothesized. In AM symbiosis in this and a previous study, it was described that the function of SymRK is restricted to the epidermis. Once fungal hyphae surpass the epidermis, possibly by using small lesions, arbuscles can be formed (Demchenko et al. 2004). Possibly, a similar mechanism comes into play for Tomato SymRK in the root systems displaying a normal nodule formation: Once epidermal infection threads successfully reach the cortex becoming cortical infection threads, fully infected and functional nodules can form. In the swellings though, no infection threads in the cortex can be observed, possibly due to the lack of epidermal infection threads, which are missing in swellings. These speculations however need to be substantiated by future experimental data.

Finally, there is a possibility that SymRK influences the interaction between *Agrobacterium rhizogenes* and the host plant. Agrobacteria and Rhizobia can be grouped into the same clade and their historic separation was mainly justified by differences in lifestyle rather than phylogenetic data such as DNA sequences (Willems and Collins 1993). If the *symrk* mutant plants have also a reduced capacity to interact with rhizobia this can influence the data from any hairy root experiment. To minimize the risk as much as possible, all roots were screened by a co-transformed GFP marker and non-transformed roots were removed before any experiment.

Comparison of Markmann and colleagues (2008) and this study

The RNS infection phenotype in the study of Markmann and colleagues (2008) and our study is substantially different: they did neither observe any nodule formation, nor swelling formation in mutant roots transformed with Tomato SymRK under the control of the Lotus SymRK promoter upon inoculation with *M. loti* MAFF *Ds*Red. This is not caused by the use of distinct mutant alleles, as in this study, I observed similar RNS phenotypes in roots of the *symrk-3* null mutant and the *symrk-10* point mutation mutant used previously (Markmann et al. 2008). The *symrk-10* mutant could even be complemented to a higher degree using Tomato SymRK. The growth conditions of *L. japonicus* however differed between the work of Markmann et al. and this study: Whereas Markmann and colleagues used expanded clay (Seramis®) and plastic

boxes (Markmann et al. 2008), in our study, sand-vermiculite and glass containers were used. A recent study on *Lotus sp.* with sub-compatible bacteria revealed a strong impact of humidity and substrate on the type of interaction strength (Yen-Yu Lin, personal communication). The different growth conditions used for the experiments might therefore be the reason for the different outcome.

Another difference potentially leading to the contrasting observations lies in the promoter sequences. The authors of that paper used topoisomerase-based (TOPO) cloning (Markmann et al. 2008) instead of Golden Gate cloning used in this work (Markmann et al. 2008). To make the SymRK promoter compatible with a Golden Gate based cloning strategy, several restriction sites of BpiI and BsaI were removed by site directed mutagenesis. This potentially created a cryptic cis-regulatory element and thus increased the expression strength slightly, but possibly enough to change the outcome of roots transformed with Tomato SymRK. In this context, overexpression of Lotus SymRK in *L. japonicus* roots leads to spontaneous nodule formation as described above (Ried et al. 2014). Thus, the amount of receptor present can make a difference in signaling output. Overexpression leading to spontaneous formation of root organs originating from cortical root cells such as lateral roots, nodule primordia, and nodules is common among nodulation genes such as *NIN*, *NF-YA1*, *Cyclops*, *NFR5* (Soyano et al. 2013; Ried et al. 2014; Singh et al. 2014). In receptor-like kinase overexpression contexts a possible mechanism is a forced interaction with downstream signaling interaction partners as these proteins usually co-localize in so called membrane nanodomains (Bücherl et al. 2017). Ried and colleagues proposed a forced interaction of SymRK with LysM-domain receptors (Ried et al. 2014). For the anchoring of the symbiotic receptors to their nanodomain, the remorin protein SYMREM and FLOT4 play an important role (Liang et al. 2018). They might thus also play a role in the formation of spontaneous nodules by overexpression of receptors. A stabilization of the receptor complex in *symrk-10* mutants by the presence of a kinase dead native SymRK may be an explanation for the observed increase in nodule formation in Tomato SymRK transformed roots compared to the *symrk-3* mutant. Another explanation for the different outcomes could be differences in the mutant background. Both mutants stem from an EMS mutagenesis screen, so most likely more than one gene is mutated (Perry et al. 2003).

The role of SymRK ubiquitination

The formation of nanodomains plays an important role in stabilizing the symbiotic receptors in the plasma membrane and prevent their recycling (Liang et al. 2018). The intracellular uptake of SymRK is thus important for its function and is induced by Rhizobia (Dávila-Delgado et al. 2023). This uptake is proposed to be dependent on E3- Ubiquitin-ligases (Den Herder et al. 2008, 2012; Liu et al. 2018; Dávila-Delgado et al. 2023). E3-ligases transfer one or several ubiquitin proteins to the lysine side chain in the target protein (Mazzucotelli et al. 2006).

Membrane proteins targeted for endocytosis are marked by two features: A conserved motif consisting of a tyrosine (Y), two random amino acids (XX) and a bulky hydrophobic aminoacid (Φ) (Ohno et al. 1995; Liu et al. 2020) and a phosphorylation site in its close vicinity (Robatzek et al. 2006). For SymRK, such a region has been identified in the intracellular juxtamembrane region of *Phaseolus vulgaris* (Dávila-Delgado et al. 2023). The intracellular uptake of SymRK is dependent on the phosphorylation of the threonine residue at the position 598 (T598) (Dávila-Delgado et al. 2023), which was identified to play an important role for SymRK function also in *L. japonicus* (Yoshida and Parniske 2005). Even though, the presence of T598 is essential for SymRK function in RNS and AM, ubiquitin-ligases have only been described to play a role in RNS (Den Herder et al. 2012; Liu et al. 2018). A potential difference in ubiquitination pattern could therefore be the reason for a difference between Tomato SymRK and Lotus SymRK.

In my experiments, a Lotus SymRK containing two point mutations in lysine residues exhibited a slightly reduced number of nodules with an increase in primordia and primordia-like structures. The SymRK version with the double point mutation might be slightly impaired in its signaling capacity. The single point mutations of Lotus SymRK though did not exhibit a difference in the formation of nodules, primordia or swellings. The amino acid change in the juxtamembrane domain of Tomato SymRK did not alter its function compared to wildtype Tomato SymRK neither. When a lysine in a predicted ubiquitination site at the C-terminus of Tomato SymRK was changed to the amino acid present in Lotus SymRK at the same position, the protein was not functionable anymore: neither were swellings visible after inoculation with *M. loti*, nor were arbuscules formed after inoculation with *R. irregularis*. This indicates that the amino acid seems to play an important role for general Tomato SymRK function. That change of amino acid might impair the function in different ways. Possibly, it does not fold correctly any more, it does not pass protein quality control or is impaired in cellular trafficking due to its different properties.

However, these experiments were designed and performed based on the ubiquitination predictions from UbPred, where known yeast ubiquitination sites were used for model training (Radivojac et al. 2010). Ubiquitination sites in plants may differ from yeast and be poorly predicted by the tool. Taken together whereas so far, no indication for a role of ubiquitination in the function of Lotus SymRK and Tomato SymRK was observed, an important difference cannot be excluded at this stage.

Another post-translational modification, which can play an important role in the signal cascades of receptor-like kinase, is the addition of a SMALL UBIQUITIN-LIKE MODIFIER (SUMO), as known for the receptor-like kinases FLS2 and BRASSINOSTEROID INSENSITIVE1 (BRI1) (Orosa et al. 2018; Naranjo-Arcos et al. 2023). Future experiments can reveal whether this

modification might mediate the differences between Lotus SymRK and Tomato SymRK signaling.

Autoregulation of nodulation and excessive swelling formation

Autoregulation of nodulation is a mechanism that stops the infection and organogenesis of new nodules, once a certain number of nodules has formed (Ferguson et al. 2019). The formation of many swellings on the same root in most Tomato SymRK expressing roots implies that this type of regulation is impaired in these roots. However, few root systems transformed with Tomato SymRK developed fully infected nodules in an amount comparable to wildtype roots. The autoregulation of nodulation comprises a long-distance signaling pathway in a root-to-shoot and a shoot-to-root direction and local signal cascades (Ferguson et al. 2019; Roy et al. 2020). Several pathways are involved in the autoregulation of nodulation which include activators and repressors of infection as well as cortical cell divisions and nodule organogenesis. An important signal are CLAVATA3/EMBRYO SURROUNDING REGION-RELATED-peptides (CLE-peptides) which are released in the root and transported to the shoot upon signals like nitrate or rhizobial contact. Those can than activate LRR-RLKs, like HYPERNODULATION ABERRANT ROOT FORMATION 1 (HAR1) or CLAVATA2 (CLV2) in the shoot and leads to the suppression of nodule formation (Reid et al. 2011; Li et al. 2022). In contrast, the C-TERMINALLY ENCODED PEPTIDE (CEP), which is produced at nitrogen deficiency status and binds to the COMPACT ROOT ARCHITECTURE 2 (CRA2)-receptor in the shoot, promotes rhizobial infection and nodule formation in nitrogen starvation (Li et al. 2022). The suppression of nodulation via the CLE/HAR1 pathway is mediated via the degradation of the microRNA miR2111. Thus the expression of TOO MUCH LOVE (TML), the target of miR2111, is no longer repressed, and TML can suppress nodulation (Tsikou et al. 2018; Gautrat et al. 2020). Supposedly, in roots forming a high number of swellings, the suppression of nodulation by production of CLE-peptides does not take place, as no nitrogen is fixed and bacteria only very rarely enter the root intracellularly. Therefore, the miR2111 is not degraded and the roots stay primed for response to bacterial signals. Probably, in addition, the positive regulation via the CEP/CRA2-pathway is also active and further increases the symbiotic root responsiveness. In those roots, that form infected nodules, their number is unchanged to wildtype roots suggesting functional autoregulation of nodulation.

Interplay of SymRK and the plant immune system

Roots expressing Tomato SymRK display a high density of Rhizobia on the root surface, or the first cell layer of the epidermis. Especially in the histological section, it is obvious, that in

contrast to a genuine primordium, the bacteria do not concentrate in small spaces but cover the whole root structure.

The SymRK protein is present in the plasma membrane of root hairs of *Phaseolus vulgaris* (Dávila-Delgado et al. 2023). Even though, in contrast to *nfr1* and *nfr5* mutants, epidermal root hair cells of *symrk* mutants still respond to Nod-factor, bacterial or fungal entry into these cells is not possible in the *symrk* mutant background (Demchenko et al. 2004; Miwa et al. 2006). Strikingly, root hair infection threads occurred more frequently than infected pink nodules in roots transformed with Tomato SymRK. To allow the intracellular uptake of Rhizobia, there is the necessity to downregulate the plant immune system (Gourion et al. 2015). This process is dependent on the presence of SymRK (Feng et al. 2021). The histological section of a swelling also exhibited a high density of *M. loti* on the root surface, or the outer epidermal cell layer. This could be due to an interference of the immune system not allowing the bacteria to proceed to inner cell layers, like the cortex. So possibly, in Tomato SymRK expressing roots, the downregulation of the immune system is not efficient enough to allow efficient bacterial entry to the inner root layers such as the cortex and pericycle. The downregulation of the plant immune system is dependent on the interaction of SymRK with the co-receptor BAK1 (Feng et al. 2021). BAK1 is known to be a co-receptor for many receptors in plants, of which the immune receptor FLS2 and the brassinosteroid receptor BRI1 are the most prominent ones (Li et al. 2002; Nam and Li 2002; Chinchilla et al. 2007). This implies a dual function of BAK1 in immunity and development, both necessary for RNS, with rhizobial uptake into roots and nodule organogenesis.

The intracellular domain of SymRK and its function

One *symrk* mutant, that resembles the phenotype observed in Tomato SymRK is *symrk-14.* This mutant also exhibits many irregularly infected primordia and rare events of infected nodule formation (Kosuta et al. 2011). This mutant has a point mutation in the MLD-LRR-RLK specific GDPC domain, linking the MLD to the LRR domain (Kosuta et al. 2011). Together with the phylogenetic data showing a difference in the number of LRR-domains between Tomato SymRK and Lotus SymRK, the hypothesis of a reduced function of the extracellular domain of Tomato SymRK arose (Markmann et al. 2008). But results of Martina Ried and this study are pointing strongly towards a functional conservation of the extracellular domain of SymRK and a functional differentiation of the intracellular domain and partially the transmembrane domain. Additionally, a study using a similar domain swap approach with an Arabidopsis SymRK homologue came to the same conclusion (Li et al. 2018). This implies, that an interaction with an RNS-specific protein might be impaired in the Tomato SymRK intracellular domain with potential involvement of the transmembrane domain. However, trying to pinpoint the

subdomain of the intracellular domain responsible for the sub-functionalization in RNS was not successful by using the domain swap approach. The results rather point towards a more important role of the transmembrane domain as already indicated by slight differences in complementation efficiency of previous whole domain swap experiments. Interestingly, the presence of at least two parts of the intracellular domain of Lotus SymRK increased the formation of infected nodules, but decreased the total number of nodules. These results imply that there might be an interaction partner necessary to establish RNS but not AM, that can only interact with SymRK when a certain percentage of the interaction surface is present.

The evolution of LysM-receptor intracellular domains is different from SymRK

A SymRK interactor, that is known to use the intracellular and transmembrane domain for the interaction is *Lj*NFR5 (Madsen et al. 2003; Antolín-Llovera et al. 2014b). In addition, the gene is dispensable for the interaction with *R. irregularis* as *nfr5* mutants have no impairment in AM formation (Madsen et al. 2003; Perry et al. 2003). However, the intracellular and transmembrane domain of *Lj*NFR5 are functionally conserved: Swap constructs of the intracellular domain of the tomato *Lj*NFR5 homologue *Sl*LYK10 and the extracellular domain of *Lj*NFR5, necessary for Nod-factor interaction, can fully restore nodulation in *nfr5-2* mutants (this work and (Seidler 2017). A similar approach with the rice homologue of NFR5 (*Os*RLK2/*Os*NFR5) led to the same conclusion (Miyata et al. 2016). A FLIM-FRET interaction assay using the intracellular domain of *Lj*NFR5 with Lotus SymRK and Tomato SymRK revealed that there is no difference in interaction strength (Seidler 2017). However, the extracellular domains of *Lj*NFR5 and *Sl*LYK10 perform distinct functions, thus expression of *Sl*LYK10 cannot complement the *nfr5-2* mutant in presence of Rhizobia. Along this line, it was also not possible to obtain spontaneous nodules by *Sl*LYK10 overexpression in *L. japonicus* wild type roots. This suggested that the downstream root nodule organogenesis signaling is not activatable by an overaccumulation of the tomato receptor-like kinase. In contrast, an overaccumulation of *L. japonicus* receptor-like kinases or even only their intracellular domains is sufficient to trigger nodule organogenesis even in the absence of any symbiont (Ried et al. 2014; Saha et al. 2014).

Similar to the intracellular domain and transmembrane domain of *Sl*LYK10, the intracellular domain of *Sl*LYK1 (formerly known as *Sl*CERK1) can also still transmit a signal for nodule organogenesis in *L. japonicus* roots, when swap constructs with the extracellular domain of *Lj*NFR1 are used. The efficiency of the complementation cannot reach the level of complementation achieved by the native *Lj*NFR1. Even the complementation of the *nfr1-1* mutant with the native *Lj*NFR1 does not lead to the formation of nodules in all the transformed plants. In another study, the percentage of nodulated plants after hairy root transformation with

a promoter of similar length than in this study (about 3 kb) was about 60% (Radutoiu et al. 2003), which is only slightly higher than in this study. A further reduction could have many causes, e.g. the adaptation of the promoter for Golden Gate cloning as discussed above. In the root systems with nodule formation this effect might be balanced by introduction of several copies during *A. rhizogenes* transformation, increasing the protein amount at the membrane above the threshold for RNS (Ried et al. 2014). For the swap construct *Lj*NFR1ED-*Sl*LYKTMID, the number of nodulating plants as well as the number of nodules per plant was further reduced. Thus, the presence of the transmembrane domain cannot increase the complementation capacity of a swap construct with *Sl*LYK1 compared to a swap construct with the kinase domain only (Miyata et al. 2014). After the conclusion of this experiment, a study suggested that in tomato four paralogues of *Lj*NFR1 exist allowing functional diversification and an AM phenotype was observed with SlLYK12 (Liao et al. 2018). In contrast to rice, where *Os*CERK1 has a dual function in AM symbiosis and immunity (Miyata et al. 2014), in tomato, there are different roles for the *Lj*NFR1 paralogues. *Sl*LYK12 among the NFR1 paralogues takes over the main function in AM symbiosis, whereas *Sl*LYK1 and *Sl*LYK13 are mainly associated with immune responses (Liao et al. 2018). Therefore, possibly a domain swap experiment with the transmembrane domain and the intracellular domain of *Sl*LYK12 and the extracellular domain of *Lj*NFR1 could lead to an increased complementation capacity.

Other interactors of SymRK might have co-evolved for RNS

As already mentioned above, SYMREM1 plays an important role in the stabilization of membrane nanodomains, as well as large scale membrane conformations, which are both necessary for rhizobial infection (Liang et al. 2018; Su et al. 2023). Additionally, SYMREM1 is described to be an interactor of SymRK (Lefebvre et al. 2010). Tomato SymRK might be affected in its interaction with SYMREM1 ultimately leading to an impaired interaction with Rhizobia.

A recent study presents *Lj*BAK1 as an additional interactor of SymRK (Feng et al. 2021). The authors propose a downregulation of BAK1 function by SymRK in order to suppress an immune response against the bacteria entering the cell (Feng et al. 2021). This study however did not investigate any function of this interaction in AM symbiosis. An impaired interaction with BAK1 could explain, why the infection with bacteria is compromised in *symrk* mutant roots expressing the intracellular domain of Tomato SymRK. This is relevant as this study reported a weak, but consistent MAP-kinase activation and defense marker gene induction by rhizobia, even as they lack a flagellin22 motif (Feng et al. 2021). This hypothesis could be substantiated by determining plant immune hallmarks such as reactive oxygen species burst, defense marker

gene induction and especially MAP-kinase phosphorylation (Monaghan and Zipfel 2012) in mutants expressing Tomato SymRK and Lotus SymRK and domain swap constructs.

Conclusion

In summary, the data of this thesis suggest a more complex evolution of SymRK than of other receptor-like kinases. S*ymrk* mutant roots complemented with Tomato SymRK exhibited root hair responses, abundant swelling formation, and, in rare cases, infection thread formation and functional nodule formation. To identify one responsible domain or amino acid motif that effectively restores the complementation capacity of Tomato SymRK was challenging. When swapping the intracellular domain, transmembrane domain and extracellular domain, it can be clearly observed that the intracellular domain plays an essential role for improved, complementation capacity, but a follow up experiment with more refined swaps could not further pinpoint a subdomain of the intracellular domain. Instead, its results rather suggested an importance of the complete intracellular domain and confirmed that the intracellular domain alone is not sufficient for the neofunctionalization for RNS. Differences in ubiquitination sites of the two SymRK versions were detected *in silico*, but so far, a role could not be verified experimentally. In contrast, the adaptation of the LysM-receptor-like kinases *Lj*NFR5 and *Lj*NFR1 is much clearer: The extracellular domain of *Lj*NFR5 differs from the counterpart in *S. lycopersicum Sl*LYK10 and is able to sense Nod-factor instead of Myc-factor, whereas the intracellular domain is functionally conserved. A complementation of *nfr5-2* mutant roots with *Sl*LYK10 does not lead to any symbiotic reaction of the roots, however a swap construct with the extracellular domain of NFR5 and the intracellular domain of *Sl*LYK10 can fully complement the mutant phenotype. In a comparable experiment, the intracellular domain of *Sl*LYK1 could complement the *nfr1-1* mutant partially, despite a potential functional differentiation of the paralogues.

Material and Methods

Media

The composition of FP medium (Fahraeus 1957; Tóth et al. 2016), Hoagland medium (Hoagland and Arnon 1938) and TY medium (Beringer 1974) were described previously. All other media that were used in this study are listed in Table 2.

Table 2: Used culture media and their composition

Component	Final concentration	Comment
LB-Medium		
Bacto.Trypton	10 g/L	-
Bacto Yeast Extract	5 g/L	-
NaCl	10 g/L	-
Bacto agar	0.8 %	-
Ultrapure water	Fill up	-
B5 medium		
m-inositol	100 mg/L	-
Pyridoxine HCl	1 g/L	-
Nicotinic acid	1 g/L	-
Thiamine	10 g/L	-
Sucrose	2 %	If necessary
Cefotaxime	300 mg/L	If necessary
Ultrapure water	Fill up	-
FAB media (low nitrate)		
$MgSO_4$ *7 H_2O	500 µM	-
KH_2PO_4	250 µM	-
KCl	250 µM	-
$CaCl_2$ *H_2O	250 µM	-
KNO_3	100 µM	-
Fe-EDDHA	25 µM	-
H_3BO_3	50 µM	-
$MnSO_4$ *H_2O	25 µM	-
$ZnSO_4$ *7 H_2O	10 µM	-
Na_2MoO_4 *2 H_2O	0.5 µM	-
$CuSO_4$ *5 H_2O	0.2 µM	-
$CoCl_2$ *6 H_2O	0.2 µM	-
MES-KOH buffer pH 5.7	2 µM	-

Component	Final concentration	Remark
Adjust to pH 5.7	-	If necessary
FAB media (low phosphate)		
$MgSO_4$ *7 H_2O	500 µM	-
KH_2PO_4	25 µM	-
$CaCl_2$ *H_2O	250 µM	-
KNO_3	1500 µM	-
Fe-EDDHA	25 µM	-
H_3BO_3	50 µM	-
$MnSO_4$ *H_2O	25 µM	-
$ZnSO_4$ *7 H_2O	10 µM	-
Na_2MoO_4 *2 H_2O	0.5 µM	-
$CuSO_4$ *5 H_2O	0.2 µM	-
$CoCl_2$ *6 H_2O	0.2 µM	-
MES-KOH buffer pH 5.7	2 µM	-
Adjust pH to 5.7	-	If necessary

Plant, fungal and bacterial material

***Lotus japonicus* seeds:**

Lotus japonicus ecotype Gifu B-129 wildtype, *symrk-3*, and *symrk-10* mutants (Handberg and Stougaard 1992; Stracke et al. 2002; Perry et al. 2003) were propagated in the ”Gewächshauslaborzentrum Dürnast” of the Technical University of Munich.

Fungi:

Rhizophagus irregularis DAOM 197198 (Agronutrition, Toulouse, France) was used for arbuscular mycorrhiza inoculation.

Bacteria:

Mesorhizobium loti strain MAFF303099 with *Ds*Red as a marker (Maekawa et al. 2008) was stored in glycerol stocks at -80 °C and used for *L. japonicus* inoculation. For hairy root transformation of *L. japonicus* roots, a glycerol stock from the *Agrobacterium rhizogenes* strain AR1193 was used (Offringa et al. 1986).

Cloning

Constructs were obtained by Golden Gate cloning as described previously (Binder et al. 2014) with adaptions to the cut-ligation protocol as described (Chiasson et al. 2019). Primers from this study are listed in Table 3, all plasmids from this study are listed in Table 4.

Table 3: Primer used in this study:

Experiment	Name	Sequence
NFR5/LYK10 domain swap		
MR346	in fwd TMID SlLYK10	AG gaagac AA AATC CAAGCATGGATGGATAG
MR348	in revTMID NFR5	AA gaagac GA AAtc CAGCATTCATCTTCTGG
MR359	NFR5 fwd	AC GAAGAC GG TACG GGTCTC c CACC atgGCTGTgTTCTTTCTTACC
MR363	NFR5 rev	TG GAAGAC GG CAGA GGTCTC a CCTT ACGTGCAGTAATGGAAGTC
MR302	SlLYK10 fwd	GT GAAGAC AT TACG GGTCTC C CACC atgGTAGTTCCTCTTGTGTCC
MR304	SlLYK10 mut1 fwd	AT GAAGAC TT tAAC CTCTGTTTTCTTGC
MR305	SlLYK10 mut1 rev	GA GAAGAC AG GTTa AAGACGATATCTGTC
MR357	SlLYK10 rev	CT GAAGAC AA CAGA GGTCTC T CCTT ACGTGCTATTACCGGAC
LjSymRK/SlSymRK domain swaps		
MR254	out fwd SlSYMRK ED	AT gaagac CC tacgggtctc G cacc ATGGAAGTAGATAATTGCTGG
MR255	in rev SlSYMRK ED	TG gaagac CA GACG ACAGAGATAACAATTGC
MR256	out fwd LjSYMRK ED	AC gaagac GG tacgggtctc A cacc ATGATGGAGTTACCAGCTAC
MR257	in rev LjSYMRK ED	TG gaagac CA gACg AAAAGAACTCCAAAAGC
MR258	in fwd SlSYMRK ID	TT gaagac GT CGTC TGCCTCTTCAAAAGACG
MR259	out rev SlSYMRK ID	GT gaagac CC caga ggtctc A cctt CCTTGGTTGTGGAGG
MR260	in fwd LjSYMRK ID	GA gaagac TT cgtc TGCCGCTACAGACAAAAATTAATTC
MR261	out rev LjSYMRK ID	TG gaagac GG caga ggtctc T cctt TCTCGGCTGTGGGTGAG
MR262	in rev LjSYMRK SP	GT GAAGAC CC TTCC GTTGCAGAAGCTGATCCGA
MR263	in rev SlSYMRK SP	TA GAAGAC CC TTCC TGTGCAAAGGCAGATTGT
MR264	in fwd LjSYMRK deltaect fits to SP	AC GAAGAC GT GGAA TATGGAAGATGCAAAGG
MR265	in fwd SlSYMRK deltaect fits to SP	GC GAAGAC GC GGAA AAAGGGATGGCTAATGT
MR266	in fwd LjSYMRK deltaect fits to ect	TA GAAGAC AC AGAT TATGGAAGATGCAAAGG
MR268	in rev LjSYMRK ect	AT GAAGAC TA ATCT GTATTGATTAGTGAACTG

MR270	in fwd SlSYMRK deltaect fits to ect	AT GAAGAC AC AGAt AAAGGGATGGCTAATGT
MR271	in rev SlSYMRK ect	CC GAAGAC TT aTCT GTTGTAATATTAGATTCT
Ubiquitination site mutations		
IS44	LjSYMRK Ubi1 fwd	TTgaagacGCAtgGATGATTTCTTCATAAAGTCCG
IS45	LjSYMRK Ubi1 rev	AAgaagacATCcaTGCTTGGCAAAGAGAAAATTATATctac
IS46	LjSYMRK rev with Ubi2	TGgaagacGGcagaggtctcTccttTCTCGGCTGTGGGTGAGACAAGGACTtTGTTGTG
IS63	SlSYMRK Ubi1 fwd	CAgaagacAGCAaGGATACTACCATG
IS64	SlSYMRK Ubi1 rev	TGgaagacTCCtTGCTTGGTACTGAG
IS65	SlSYMRK Ubi2 rev	GTgaagacCCcagaggtctcAccttTTCCTTGGTTGTGGAGGGGCTGGTTgTGAGAGG
Intracellular subdomain swap		
IS55	LjSYMRK juxtamembrane in rev	AAgaagacTTCACCTATCAAGGTTTTGTACCTCTC
IS88	SlSYMRK juxtamembrane in rev new	AAgaagacTTCACCTATCAAAGTTTTGTAGTTCTGAG
IS57	LjSYMRK conserved KD in fwd	AAgaagacTAGGTGAAGGAGGGTTTGGCTCTGTTTACAGG
IS58	SlSYMRK conserved KD in fwd	AAgaagacTAGGTGAAGGTGGCTTTGGATCCGTTTACCG
IS59	LjSYMRK conserved domain in rev	ATgaagacCTCCTTCCTGTGGAGCATATTTTGAGAAACC
IS60	SlSYMRK conserved domain in rev	ACgaagacCCCCTTCTTGAGATGCATATTTTGAAAATCC
IS61	LjSYMRK less conserved KD in fwd	GCgaagacGGAAGGAGATAGTTATGTCTCCCTTGAAGTAAGAGG
IS62	SlSYMRK less conserved KD in fwd	GCgaagacAGAAGGGGATAGTGGTACTTCTTTAGAAGTAAGG
Domain swap *Lj*NFR1/*Sl*LYK1 (formerly known as *Sl*CERK1)		
MR300	SlCERK1 fwd	AA GAAGAC TT TACG GGTCTC G CACC atgTTTGAATCCAGGCCAAG
MR306	SlCERK1 mut 1 fwd	TC GAAGAC TT gAGT GTTGGCTTATTTGTTATCC
MR307	SlCERK1 mut 2 fwd	TC GAAGAC GA gGAC CACCTCCATCAGTATGG
MR308	SlCERK1 mut 3 fwd	GA GAAGAC AG tCAA ATGCAGAAGAACATTCG
MR309	SlCERK1 mut 4 fwd	TG GAAGAC GT gTTC AACATTCTTAAATGG
MR310	SlCERK1 mut 1 rev	AT GAAGAC AC ACTc AAGACGAGGAAAGATGC

MR311	SlCERK1 mut 2 rev	GA GAAGAC TG GTCc TCCGATCTGAGCAGC
MR312	SlCERK1 mut 3 rev	TC GAAGAC AT TTGa CTTCCCTGTCCTTG
MR313	SlCERK1 mut 4 rev	AT GAAGAC TT GAAc ACCCTGTCCAAAACC
MR352	SlCERK1 in fwd TMID	GG gaagac CC tCCG CTGCCAACAAG
MR356	SlCERK1 rev	CG GAAGAC TG CAGA GGTCTC G CCTT CCTTCCAGACATGAGG
MR358	NFR1 fwd	AC GAAGAC GG TACG GGTCTC G CACC atgAAGCTAAAAACTGG
MR355	in rev LysMD NFR1	TG gaagac AA cGGA ACATAGACTCC
MR362	NFR1 rev	TG GAAGAC GG CAGA GGTCTC c CCTT TCTCACAGACAGTAAATTTATG

Table 4: Plasmids used in this study

	Level	Transformation marker	gene of interest	Comments	created by
IS3.1	Level III	*pro35S:eGFP*	*proNFR5:NFR5-cmyc*	Used for Figure 9	Isabel Seidler
IS3.2	Level III	*pro35S:eGFP*	*proNFR5:NFR5ED/LYK10TMID-cmyc*	Used for Figure 9	Isabel Seidler
IS3.3	Level III	*pro35S:eGFP*	*proNFR5:SlLYK10-cmyc*	Used for Figure 9	Isabel Seidler
IS3.10	Level III	*proUbi:eGFP*	*proNFR1:NFR1 -his*	Used for Figure 11	Isabel Seidler
IS3.11	Level III	*proUbi:eGFP*	*proNFR1:NFR1ED/CERK1TMID-his*	Used for Figure 11	Isabel Seidler
IS3.12	Level III	*proUbi:eGFP*	*proNFR1:CERK1-his*	Used for Figure 11	Isabel Seidler
IS3.13	Level III	*pro35S:eGFP*	*proLjUbi:LYK10-cmyc*	Used for Figure 10	Isabel Seidler
IS3.14	Level III	*pro35S:eGFP*	*proLjUbi: NFR5-cmyc*	Used for Figure 10	Isabel Seidler
IS3.18	Level III	*pro35S:eGFP*	*proSYMRK:LjSYMRK Ubi1/2-HA*	Used for Figure 7 - supplementary Figure S2	Isabel Seidler
IS3.20	Level III	*pro35S:eGFP*	*proSYMRK:ev*	Used for Figure 8	Isabel Seidler
IS3.23	Level III	*pro35S:eGFP*	*proSYMRK:Sl ED - Lj JXT - Lj cons - Sl c-tail-HA*	Used for Figure 8	Isabel Seidler
IS3.25	Level III	*pro35S:eGFP*	*proSYMRK:Sl ED - Lj JXT - Sl cons - Sl c-tail -HA*	Used for Figure 8	Isabel Seidler
IS3.27	Level III	*pro35S:eGFP*	*proSYMRK:Sl ED - Lj JXT - Sl cons - Lj c-tail-HA*	Used for Figure 8	Isabel Seidler
IS3.28	Level III	*pro35S:eGFP*	*proSYMRK:Sl ED - Lj JXT - Lj cons - Lj c-tail-HA*	Used for Figure 8	Isabel Seidler
IS3.31	Level III	*pro35S:eGFP*	*proSYMRK:Lj ED - Lj JXT - Lj cons - Sl c-tail-HA*	Used for Figure 8	Isabel Seidler
IS3.33	Level III	*pro35S:eGFP*	*proSYMRK:Lj ED - Lj JXT - Sl cons - Sl c-tail-HA*	Used for Figure 8	Isabel Seidler

IS3.35	Level III	*pro35S:eGFP*	*proSYMRK:Lj ED - Lj JXT - Sl cons - Lj c-tail-HA*	Used for Figure 8	Isabel Seidler
IS3.36	Level III	*pro35S:eGFP*	*proSYMRK:LjSYMRK-HA*	Used for Figure 8	Isabel Seidler
IS3.37	Level III	*pro35S:eGFP*	*proSYMRK:SlUbi1-HA*	Used for Figure 7	Isabel Seidler
IS3.38	Level III	*pro35S:eGFP*	*proSYMRK:SlUbi2-HA*	Used for Figure 7	Isabel Seidler
IS3.39	Level III	*pro35S:eGFP*	*proSYMRK:LjUbi1-HA*	Used for Figure 7	Isabel Seidler
IS3.40	Level III	*pro35S:eGFP*	*proSYMRK:LjUbi2-HA*	Used for Figure 7	Isabel Seidler
IS3.41	Level III	*pro35S:eGFP*	*proSYMRK:Sl ED - Sl JXT - Lj cons - Sl c-tail-HA*	Used for Figure 8	Isabel Seidler
IS3.42	Level III	*pro35S:eGFP*	*proSYMRK:SlSYMRK-HA*	Used for Figure 8	Isabel Seidler
IS3.43	Level III	*pro35S:eGFP*	*proSYMRK:Sl ED - Sl JXT - Sl cons - Lj c-tail-HA*	Used for Figure 8	Isabel Seidler
IS3.44	Level III	*pro35S:eGFP*	*proSYMRK:Sl ED - Sl JXT - Lj cons - Lj c-tail-HA*	Used for Figure 8	Isabel Seidler
IS3.45	Level III	*pro35S:eGFP*	*proSYMRK:Lj ED - Sl JXT - Lj cons - Sl c-tail-HA*	Used for Figure 8	Isabel Seidler
IS3.46	Level III	*pro35S:eGFP*	*proSYMRK:Lj ED - Sl JXT - Sl cons - Sl c-tail-HA*	Used for Figure 8	Isabel Seidler
IS3.47	Level III	*pro35S:eGFP*	*proSYMRK:Lj ED - Sl JXT - Sl cons - Lj c-tail-HA*	Used for Figure 8	Isabel Seidler
IS3.48	Level III	*pro35S:eGFP*	*proSYMRK:Lj ED - Sl JXT - Lj cons - Lj c-tail-HA*	Used for Figure 8	Isabel Seidler
MR3.11	Level III	*pro35S:eGFP*	*proSYMRK:Tomus-HA*	Used for Figure 3	Martina Ried
MR3.12	Level III	*pro35S:eGFP*	*proSYMRK:Tomato SYMRK-HA*	Used for Figures 2, 3, 4, 5, 6, 7, Fig. 7 - suppl. Fig. S2	Martina Ried
MR3.13	Level III	*pro35S:eGFP*	*proSYMRK:Lotus SYMRK-HA*	Used for Figures 2, 3, 4, 5, 6, 7, Fig. 7 - suppl. Fig. S2	Martina Ried
MR3.14	Level III	*pro35S:eGFP*	*proSYMRK:Lotato-HA*	Used for Figure 3	Martina Ried
MR3.15	Level III	*pro35S:eGFP*	*proSYMRK:Lomato-HA*	Used for Figures 2, 3	Martina Ried
MR3.16	Level III	*pro35S:eGFP*	*proSYMRK:Totus-HA*	Used for Figures 2, 3	Martina Ried
MR3.17	Level III	*pro35S:eGFP*	*proSYMRK:Lotus deltaED-HA*	Used for Figure 3	Martina Ried
MR3.18	Level III	*pro35S:eGFP*	*proSYMRK:Tomato deltaED-HA*	Used for Figure 3	Martina Ried
MR3.33	Level III	*pro35S:eGFP*	*proSYMRK:Lotus delta MLD-HA*	Used for Figure 3	Martina Ried
MR3.34	Level III	*pro35S:eGFP*	*proSYMRK:Tomato deltaMLD-HA*	Used for Figure 3	Martina Ried
MR3.35	Level III	*pro35S:eGFP*	*proSYMRK:Lomus-HA*	Used for Figure 3	Martina Ried
MR3.36	Level III	*pro35S:eGFP*	*proSYMRK:Totato-HA*	Used for Figure 3	Martina Ried
MR3.21	Level III	*pro35S:eGFP*	*proLjUbi:Lotus SYMRK-mOrange*	Used for Figure 10	Martina Ried

Phylogenetic analysis

For the phylogenetic analysis, a BLAST search (Altschul et al. 1997) in the following databases was applied using Lotus SymRK (Lj2g3v1467920.1) as a query: For sequences from *Lotus japonicus*, MG20 genome v.3.0 from LotusBase (lotus.au.dk) was used (Mun et al. 2016). Rice genome BLAST was performed on EnsemblPlants on the genome of *Oryza sativa* japonica group genome version IRGSP-1.0. The Tomato protein sequences were found on EnsemblPlants in the *Solanum lycopersicum* cv. Heinz SL3.0 version (https://plants.ensembl.org). After BLAST search, the 20 first hits of each of the species were taken for further analysis.

To align the sequences, the MAFFT server was used (http://mafft.cbrc.jp/alignment/server/) with the setting Auto. The resulting alignment was reviewed in Seaview (Gouy et al. 2010). Trimming of long gaps and totally unconserved sequence parts was performed with this program. Different stringency in the trimming of non-conserved regions resulted in very similar trees, not affecting the main result of a separation of SymRK versions in one tree branch.

For the phylogenetic tree, the webtool of CIPRES Science gateway (http://www.phylo.org/) was used (Miller et al. 2010). To get an maximum-likelihood tree, the program of Randomized Axelerated Maximum Likelihood (RAxML) version Blackbox (8.2.9) was used (Stamatakis 2014)- with the following settings: Sequence type: protein, protein substitution matrix: JTT. The resulting maxim-likelihood tree was visualized in FigTree.

For synteny analysis, the website CoGe (https://genomevolution.org/coge/), a research tool for comparative genomics, was used. For initial synteny analysis, the function of SynFind was applied, with the *M. truncatula* genome version v4 (id 22582 JCVI unmasked v4) as reference. It was compered to *L. japonicus* genome version v2.5 (id 12471 http://www.kazusa.or.jp/ unmasked v2.5) and *S. lycopersicum* genome version v3.10 (id 35173 Sol Genomics Network unmasked v3.10). The annotation Medtr5g030920.1 was used as a reference. The output of this analysis was a table with identified genes or genomic regions and the synteny score identified by the algorithm.

After obtaining the results, a GEvo-analysis on the same website was performed using the three identified chromosomal regions (Medtr5g030920.1 for *M. truncatula*, CM0177340.r2.m for *L. japonicus*, and CDS:Solyc02g091590.3.1.1 for *S. lycopersicum*) as input.

Ubiquitination prediction

For the prediction of ubiquitination sites in SymRK versions, the tool UbPred (http://ubpred.org/) was used (Radivojac et al. 2010). For the analysis, the protein sequences

of the SymRK versions of interest were entered and the algorithm calculated low, middle and high probability scores for ubiquitination sites.

Plant growth

For germination, *L. japonicus* seeds were scarified using sandpaper. The seeds were surface sterilized in 2 mL tubes by mixing them with sterilization solution (1.2% NaOCl, 0.1% SDS) and incubating them in the solution 5-8 min and inverting the tube. In sterile condition (laminar flow hood), the seeds were washed with sterile water until no foam was visible anymore (app. five times). Seeds were imbibed in sterile water for 14-17 h at room temperature on a rotation wheel. Seeds were transferred to plates with water agar (0.8%) or 0.5x B5 plates and incubated in the dark for three days. Afterwards, they were transferred to long-day conditions (16 hours light, 8 hours dark) for 3-4 days at 24 °C, 50 µE active photon flux density and 70% relative humidity. In total, the plants were 6-7 days old before hairy root transformation.

Bacterial growth conditions

Electrocompetent *A. rhizogenes* AR1193 were transformed by electroporation and used for hairy root transformation. They were grown in LB-Medium at 28 °C with respective antibiotics according to the plasmid used. *M. loti* MAFF303099 dsRed was cultivated in TY-medium containing gentamycin at 28 °C.

Hairy root transformation

For hairy root transformation, a plate of uniformly thick grown *A. rhizogenes* AR1193 containing the respective plasmid was used for each construct. The bacteria were resuspended in sterile water on the plate and the suspension was transferred to a pre-soaked sterile filter paper. The 6–7-day old seedlings were transferred to the filter paper as well with the roots and hypocotyl lying on the filter paper. Then, the roots were cut at the hypocotyl and the stems were transferred to square plates with B5-medium without sucrose under sterile conditions. The plates were transferred to dark at 18-20°C for 2 days, then transferred to long-day light conditions (16 h light, 8 h dark, 50 µE active photon flux density, 24 °C). 4-5 days after the transformation, the plants were transferred to fresh sterile plates containing B5 with 2% sucrose and cefotaxime (300 mg/mL). For three weeks, the plants were shifted to fresh sterile plates every 4-5 days. After three weeks, the transformation success was identified by screening the plants for the GFP transformation control (expressed under the control of *proUbi*-promoter) under a fluorescence stereo microscope (Leica, M165FC).

For the analysis of root hair infection or nodulation phenotypes, the plants with transformed roots were transferred into sterile glass containers from the brand Weck (Weck jars: SKU745 and SKU743), filled with 300 mL dried sand-vermiculite. The sand vermiculite was mixed with 25 mL of FP-Medium with aminoethoxyvinylglycine (AVG) mixed with *M. loti* MAFF303099 *Ds*Red OD_{600}=0.05 for figure 3 or FAB-Medium low nitrogen containing *M. loti* MAFF303099 *Ds*Red with an OD_{600} of 0.05 for all other experiments. The Weck jars were closed with micropore tape and only opened for analysis of phenotypes (19-21 days for nodulation, 7 days for root hair infection)

Chive nursing cultures were used for infection assays with *R. irregularis*. To get them, surface sterilized chive seeds were sown in sand-vermiculite and around 5000 spores of *R. irregularis* DAOM 197198 (Symplanta) were added in ¼ Hoagland medium. At 8 weeks, the germinated chive plants were cut, and the transformed *L. japonicus* seedlings were transferred to the same pots. The pots containing both the jive roots and the *L. japonicus* seedlings were watered with 20 mL of sterile FAB medium low phosphate three times per week until analysis.

Formaldehyde fixation

After harvesting the plants for nodulation and root hair infection assays from the Weck jars, the roots were thoroughly cleaned from sand and vermiculite in water. The shoots were cut, and the roots were fixed in 4% formaldehyde in 50 mM PIPES (pH 7) by vacuum infiltration three to four times for app. 15 min each. Fixed roots were washed with PIPES, pH 7 for 3 times and then transferred to 70% ethanol for 5 h, before storage in 50 mM PIPES, pH 7 at 4 °C.

Ink staining for Arbuscular mycorrhiza

After harvesting the plants for AM phenotyping, the roots were thoroughly cleaned from sand and vermiculite using tap water. The non-transformed roots, identified by the lack of GFP fluorescence of a root system were removed by cutting them under a stereo microscope (Leica, M165FC). AM fungi were stained by acetic acid ink staining as described previously (Vierheilig et al. 1998). In brief, roots were harvested in 10% KOH, boiled for 15 min, and incubated in 10 % acetic acid for 10 min. Afterwards, roots were stained with black ink and destained in 5% acetic acid for 5 min and inspected using a bright field microscope.

Phenotypic analysis and quantification

For nodulation assays, the plants were screened for transformation by screening for GFP fluorescence and nodule organogenesis by *Ds*Red fluorescence and in the white light under

the stereo microscope (Leica, M165FC). The formation of entrapments and infection threads was screened using the GFP fluorescence for transformation control and the *Ds*Red for identification of *M. loti* using an upright epifluorescence microscope (Leica DM6 B). Roots were embedded in 6% low-melting agarose, sliced into 50 µm thick sections using a vibrating-blade microtome (Leica VT1000 S), and inspected using the epifluorescence microscope.

Statistical analysis and data plots

R scripts were used for data plotting and statistical analysis (R studio software version 2022.12.0 Build 353 and R version 4.2.2). Box plots and overlapping stripcharts represent the data points at “minimum”, first quartile [Q1], median, third quartile [Q3], and “maximum” as well as all individual data points. Figures and data were organized using Adobe illustrator.

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Supplementary figures and tables

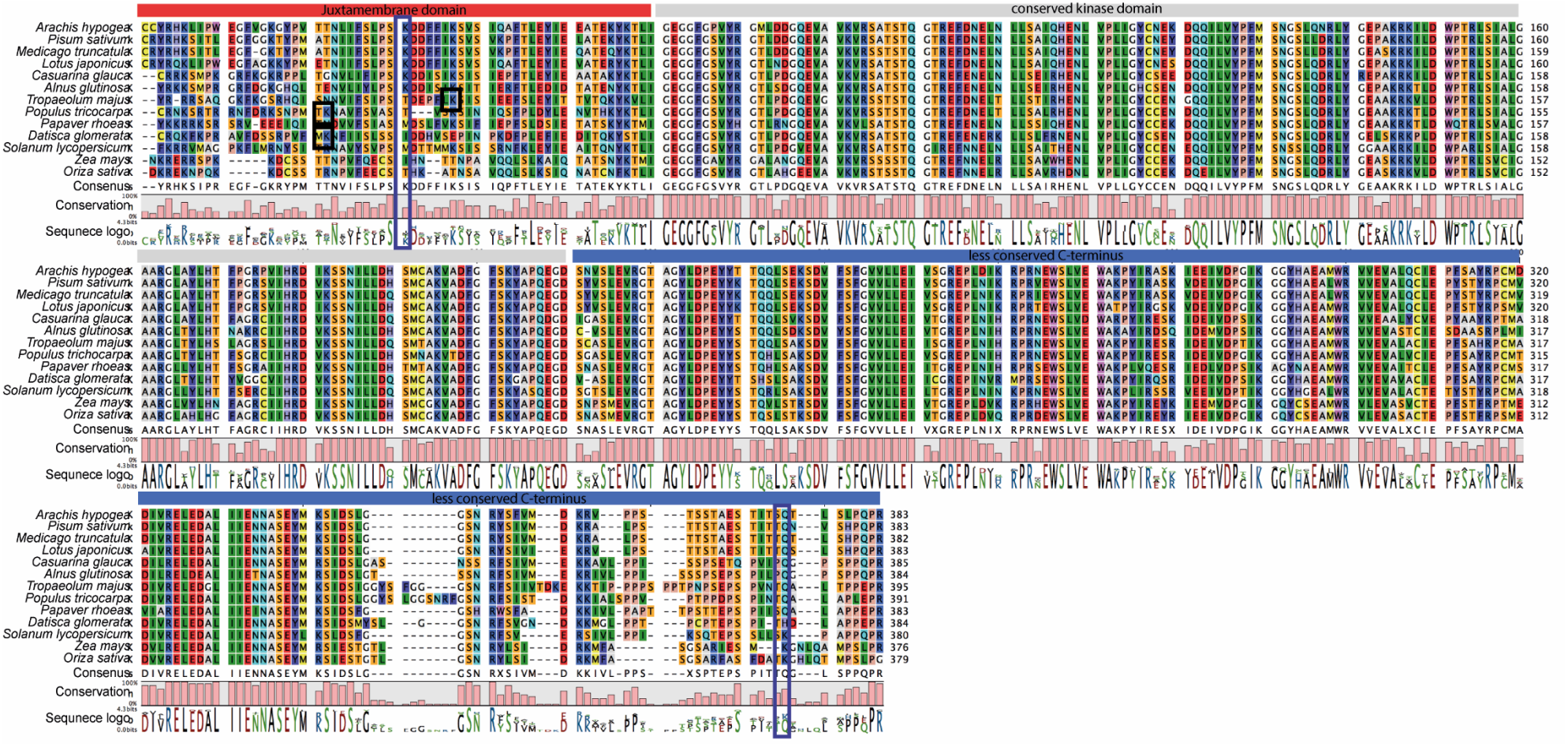

Fig. 7 - Supplementary Fig. S1: **Alignment of the intracellular domain of SymRK from several species from the rosid clade and non-rosid clade**. The intracellular domain of SymRK from *Arachis hypogea*, *Pisum sativum*, *Medicago truncatula* and *Lotus japonicus* as representatives of the Fabales, *Alnus glutinosa* and *Casuarina glauca* as representatives of the Fagales, *Datisca glomerata* as a representative of the Cucurbitales, *Tropaeolum majus* and *Populus trichocarpa* as representatives of the eurosids II, *Papaver rhoeas* and *Solanum lycopersicum* as representatives of the non-rosid dicots and *Zea mays* and *Oriza sativa* as representatives of the monocots were aligned in CLC Workbench. The red square above the alignment symbolizes the juxtamembrane domain, the grey box the conserved part of kinase domain and the blue box the less conserved part of the intracellular domain including the C-terminal tail. Blue boxes over the alignment indicate predicted Ubiquitination sites in either the rosid clade (in the juxtamembrane) with indication by black boxes of lysine (K) residues in close vicinity, if not in line, or in only outside the rosids in the C-terminus of the protein.

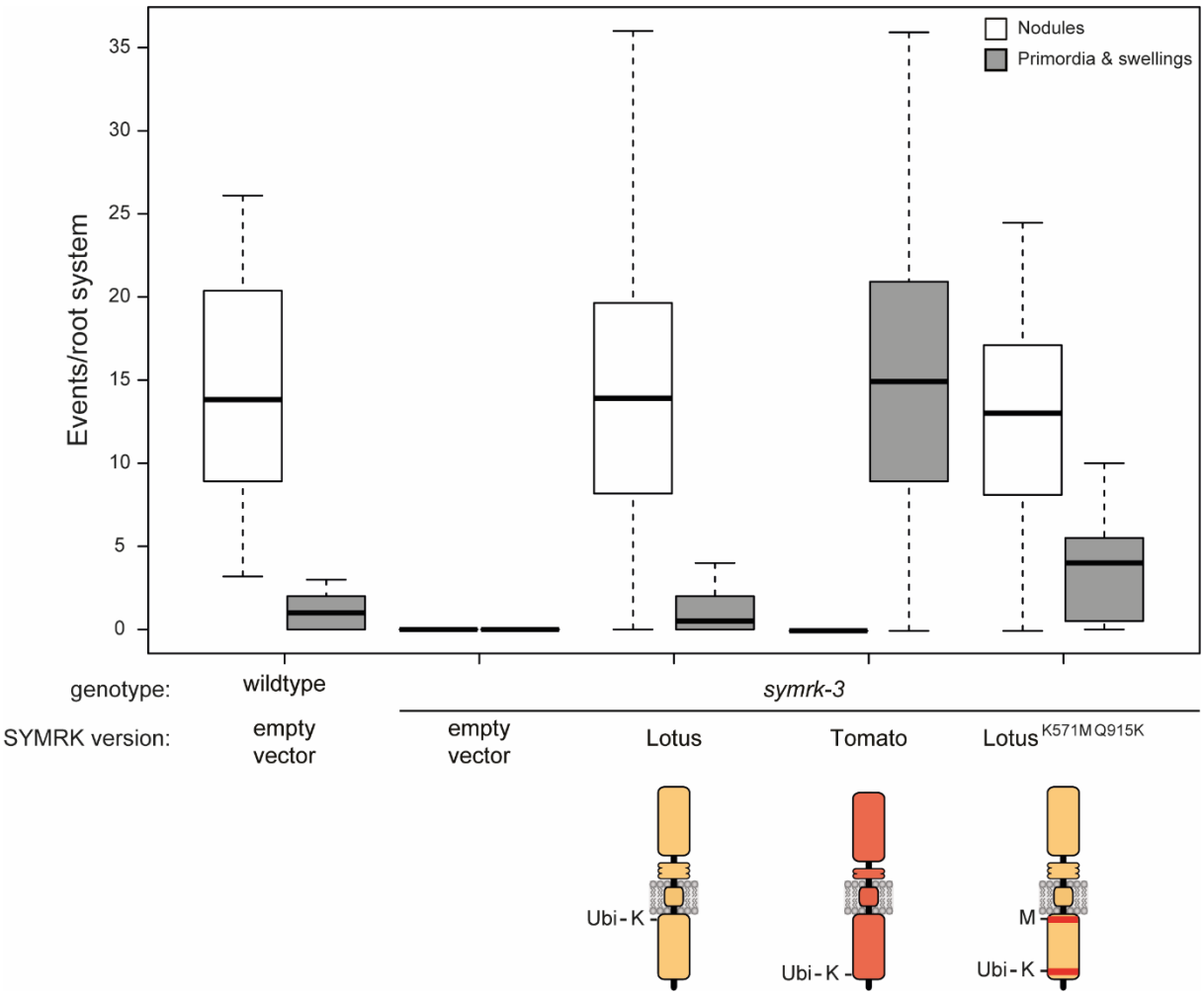

Fig. 7 - Supplementary Fig. S2: Mutation of both identified sites in Lotus SymRK lead to a slightly increased number of swellings and primordia. The following constructs were used for hairy root transformation in the *symrk-3* mutant: empty vector, Lotus SymRK, Tomato SymRK and a construct with two point mutations in the Lotus SymRK background at position 571 where lysine was exchanged for methionine and the glutamine at position 915 was replaced by lysine (Lotus^{K571MQ915K}). The constructs were under the control of the native SymRK promoter. As a control, the wildtype was transformed with empty vector. The transformed root systems were inoculated with *M. loti* MAFF *Ds*Red for 21 days. Mutant roots containing ev did not display any nodules, primordia or swellings. Mutant roots transformed with Lotus SymRK exhibited a high number of nodules and a lower number of primordia and swellings, comparable to the wildtype transformed with empty vector. Root systems transformed with Tomato SymRK displayed a low number of nodules but a high number of primordia and swellings. In mutant roots transformed with Lotus^{K571MQ915K} the number of nodules observed did not differ from the number

of nodules observed in mutant root systems transformed with Lotus SymRK, but the number of nodules and primordia was higher than in the roots transformed with Lotus SymRK but lower than in those transformed with Tomato SymRK. The boxplot represents the interquartile range of the data from first to third quartile, the solid line represents the median of the values and the whiskers represent the data range excepts outliers as classified by R default settings. All single data points are displayed as closed circles. At least 27 plants were scored per genotype. As the data are skewed towards zero values no statistical test was applied.

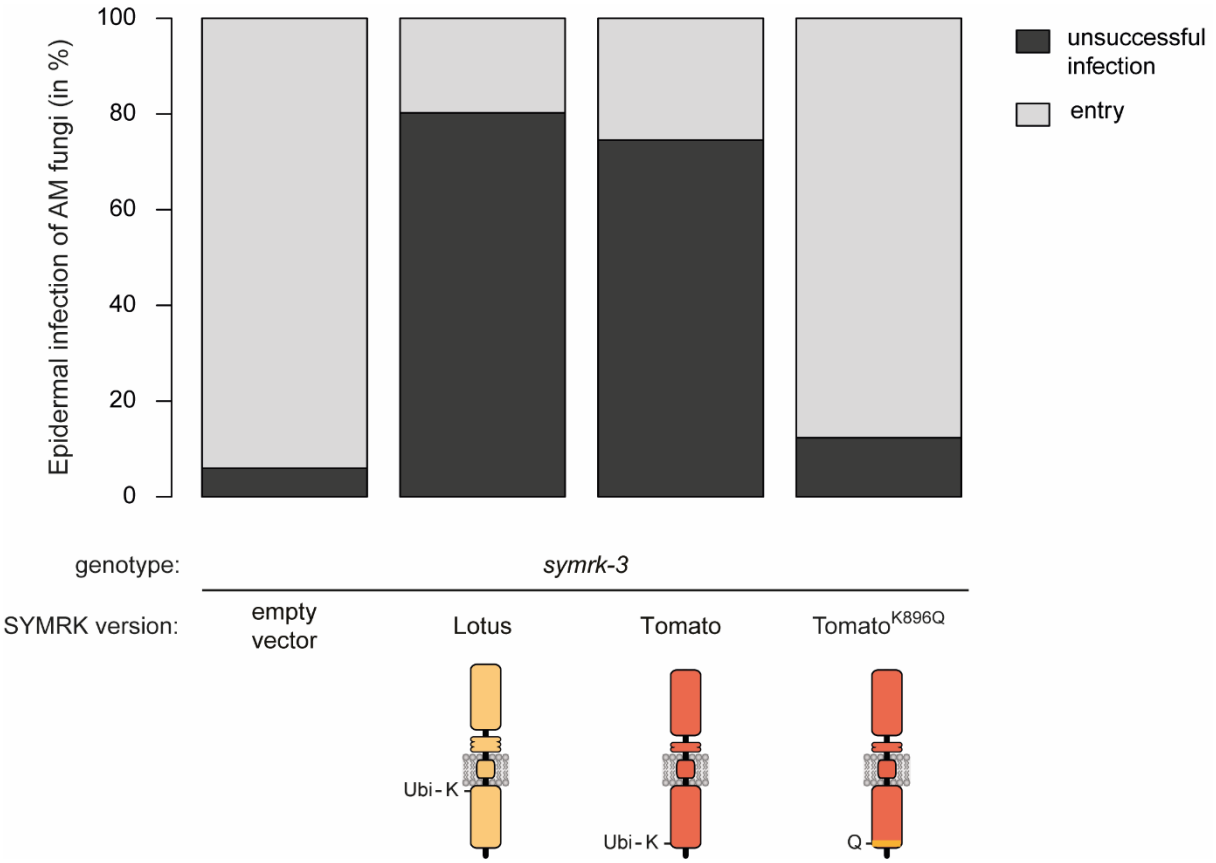

Fig. 7 - Supplementary Fig. S3: The Tomato SymRK version Tomato^{K896Q} cannot complement the AM phenotype of the *symrk-3* mutant. The *symrk-3* mutant was transformed by hairy root transformation with Lotus SymRK, Tomato SymRK and a construct with a point mutation in the Tomato SymRK background with a replacement of lysine at position 896 with glutamine (Tomato^{K896Q}) under the control of the native Lotus SymRK promoter and empty vector as control. Transformed roots were inoculated for 12 days with *R. irregularis,* that was nursed with chives before. In *symrk-3* mutants transformed with empty vector low entrance rate of *R. irregularis* could be observed as reported previously (Demchenko et al. 2004), but only attachment to the root surface. The roots transformed with Lotus SymRK or Tomato SymRK displayed a high percentage of successful infection events as described before (Fig. 2). In root systems transformed with Tomato^{K896Q}, a low number of successful infection events could be observed, similar to the *symrk-3* mutant transformed with empty vector.

Supplementary Table S1: Amino acid compositions of swap constructs used in this study

Construct name	**amino acids from *L. japonicus***	**amino acids from *S. lycopersicum***
Lotus SymRK	1-923	
Tomato SymRK		1-903
Lomus	1-503,540-923	483-520
Totato	504-539	1-483, 521-903
Totus	504-923	1-483
Tomus	540-923	1-520
Lomato	1-503	483-903
Lotato	1-539	521-903
Tomato Δ ED		1-30, 485-903
Tomato Δ MLD		1-30, 388-903
Lotus Δ ED	1-31, 505-923	
Lotus Δ MLD	1-31, 384-923	
Sl - Lj JXT	540-600	1-520, 582-903
Sl - Lj JXT - Lj KD	540-748	1-520, 730-903
Sl - Lj JXT - Lj C-term	540-600, 749-923	1-520, 582-729
Lj - Sl KD	1-600, 749-923	582-729
Lj - Sl C-term	1-748	730-903
Lj - Sl KD - Sl C-term	1-600	582-903
Sl - Lj C-term	749-923	1-729
Sl - Lj KD	601-748	1-581, 730-903
Sl - Lj KD - Lj C-term	601-923	1-581
Sl - Lj ID	540-923	1-520
Lj - Sl ID	1-539	521-903
Lj - Sl JXT - Sl C-term	1-539, 601-748	521-582, 730-903
Lj - Sl JXT - Sl KD	1-539, 749-923	521-729
Lj - Sl JXT	1-539, 601-923	521-582
*Lj*NFR5	1-595	
*Lj*NFR5 ED - *Sl*LYK10 TMID	1-239	239-617
*Sl*LYK10		1-617
*Lj*NFR1	1-623	
*Lj*NFR1 ED - *Sl*LYK1 TMID	1-219	224-626
*Sl*LYK1		1-626

Supplementary Table S2: seedbags used in this study as listed in ZopRA Plant and Seed Database

Experiment	genotype	seedbag number
Fig. 2B	symrk-3	111816, 111814, 111813, 111648, 111650, 111651, 111815
Fig. 2B	Gifu	111194
Fig. 2C	symrk-3	111807, 111808, 11809, 111810, 111811, 111812
Fig. 2C	Gifu	110893
Fig. 3	symrk-3	86983, 86926, 90309, 89484, 89482, 89483, 89389, 89401, 89402, 89400
Fig. 3	Gifu	87984, 87945
Fig. 9	nfr5-2	89386, 89051, 89015, 89306
Fig. 9	Gifu	88493
Fig. 11	nfr1-1	89357, 89358, 89353, 89500, 89501
Fig. 11	Gifu	88493
Fig. 10	Gifu	88493
Fig. 7 - suppl. Fig. S2	symrk-3	111805, 111642, 111640, 111645
Fig. 7 - suppl. Fig. S2	Gifu	110893
Fig. 6	symrk-10	92194
Fig. 6	Gifu	110894
Fig. 4, Fig. 5	symrk-3	111652, 111653, 111654, 111818, 111817, 111797, 111820, 111798
Fig. 4, Fig. 5	Gifu	111242
Fig. 8	symrk-3	111838, 111666, 111836, 111835, 111837, 111825, 111831, 111833, 111660, 111832, 111661, 111664, 111665, 111663, 111834, 114432, 114434, 114435, 114436, 114437, 115658, 115669
Fig. 8	Gifu	111242
Fig. 7	symrk-3	114438, 114430, 114431
Fig. 7	Gifu	111424
Fig. 7 - suppl. Fig. S3	symrk-3	114439, 114441, 111829
Fig. 7 - suppl. Fig. S3	Gifu	110894

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